THE BIOCHEMICAL JOURNAL

CAMBRIDGE
UNIVERSITY PRESS
LONDON: BENTLEY HOUSE
CHICAGO
The University of Chicago Press
(Agents for the United States)
BOMBAY CALCUTTA MADRAS
Macmillan
TOKYO
Maruzen Company Ltd

All rights reserved

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY
BY
C. R. HARINGTON

ASSOCIATE EDITORS
S. J. COWELL
F. DICKENS
and
F. J. W. ROUGHTON

VOLUME XXXIV 1940

PART I, pp. 1 to 779

CAMBRIDGE
AT THE UNIVERSITY PRESS
1940

PRINTED IN GREAT BRITAIN

1. THE NATURE OF SUGAR IN THE MILK AND THE CARBOHYDRATE METABOLISM OF LACTATING DIABETIC WOMEN

By KAMEL MIKHAIL DAOUD

From the Biochemical Department, Faculty of Medicine, Cairo, Egypt

(*Received 27 October 1939*)

Experiments performed by Bert [1884], Kaufmann & Hagne [1906], Porcher [1909, 1], Paton & Cathcart [1911] and Röhmann [1919] indicate that the glucose of the blood stream is the substrate for lactose synthesis by the mammary gland. As it is known that in diabetes mellitus the hyperglycaemia causes the appearance of glucose in various secretions such as saliva, sweat, bile, pancreatic secretion etc., it became of interest to investigate whether, in this condition, glucose is found in milk together with lactose or whether the mammary gland is still capable of secreting all the sugar of milk in the form of lactose. That the mammary glands, under certain conditions, can secrete unchanged glucose has been shown by Grant [1936] who injected prolactin for 2 weeks into animals which had gone completely dry.

Two cases of diabetic nursing women were available. Their milk sugar was examined for different periods during lactation. During pregnancy the two patients were receiving insulin injections whenever it was found necessary and care was taken to prevent glycosuria. It was noticed, however, that for a certain period after parturition and during lactation glucose ceased to appear in urine. The patients themselves could record the improvement in their state by the disappearance of symptoms such as thirst and polyuria despite the cessation of insulin and of the restrictions of the diet which then contained liberal amounts of carbohydrate. The weights of the patients were constant during this period of lactation. The sugar tolerance curves before pregnancy indicated moderate diabetes in case I and severe diabetes in case II (Table 1). During lactation the normal state continued for 8 months in case I and for 7 months in case II after which glucose started to reappear in the urine and the symptoms recurred.

The disappearance of glycosuria for a certain period during lactation suggested changes in carbohydrate metabolism in that condition. Some investigations were carried out on the milk, blood and urine of the two diabetic subjects in an attempt to throw some light on the nature of these metabolic changes. Further work on the latter problem is still proceeding on animals.

Experimental

The nature of sugar in the milk of diabetics

The two subjects of this investigation were suffering from typical diabetes mellitus as could be seen from the nature of their sugar tolerance (Table 1) which was worked out before pregnancy.

Sugar was present in the fasting urine in both cases and increased in amount after the ingestion of glucose.

Samples of milk were taken in both cases at different periods after parturition. The milk was always collected aseptically. The proteins and fat were precipitated

Table 1

Time	Blood sugar mg. per 100 ml. Case I	Case II
Fasting	191	247
½ hr. after ingestion of 50 g. glucose	244	341
1 ,,	254	367
1½ ,,	233	376
2 ,,	215	361

by dialysed iron and the nature of the sugar was determined in the clear filtrate. The phenylosazone of the sugar present in the filtrates obtained from the different samples was always completely soluble in hot water, and crystallized from cold water giving the yellow clusters of fine needles with the microscopic appearance characteristic of lactose phenylosazone and showing no trace of phenylglucosazone crystals. The mean M.P. of these osazones after recrystallization was 199·5°. The M.P. of pure lactosazone under the same experimental conditions was 199·9° and that of pure glucosazone 207°. The characteristics of the phenylosazone derivative of the sugar were entirely those of phenyl lactosazone. This could not be taken as conclusive evidence of the entire absence of glucose but undoubtedly indicated that if glucose was present at all it would be in a low percentage since a large excess of lactosazone might increase the solubility of a small amount of glucosazone and the characteristics of the former would not, then, be appreciably masked by the latter. For further investigation advantage was taken of the fact that yeast (*Saccharomyces cerevisiae*) can ferment glucose but not lactose. 10 ml. of every sample of milk examined were diluted with 5 ml. of water, the mixture was deproteinized and defatted by treatment with 4 ml. of dialysed iron and filtered. The precipitate was repeatedly washed with small amounts of water. The filtrate and washings, adjusted to neutrality, were concentrated in a vacuum and the volume was adjusted to 10 ml. in a measuring flask. The liquid was acidified with a little tartaric acid to inhibit the growth of bacteria which might attack lactose and cause its fermentation by the yeast. 0·5 ml. of this liquid was treated with 3 drops of a suspension of yeast in water in Lohnstein's apparatus. A control experiment was made by dissolving 20 mg. of glucose in 8 ml. of the same acidified milk filtrate to bring about a 0·25% concentration of glucose and 0·5 ml. of the latter liquid was treated with 3 drops of the same suspension of yeast. After about 5 hr. at 37° a quantity of CO_2 was evolved in the control experiment which, within the limits of experimental error, accounted for the added glucose while no evolution of gas took place in the milk filtrate alone. From this result it could be inferred that the samples of yeast used were active, that the milk filtrates did not contain anything which might inhibit the fermentative power of yeast and that glucose was absent from the samples of milk. The difference in solubility of glucose and lactose in cold absolute methyl alcohol was utilised for the confirmation of the absence of the former from the milk of lactating diabetics. Lactose is practically insoluble in methyl alcohol, while according to Trey [1901] 100 ml. of absolute methyl alcohol at 17·5° dissolve 1·25 g. of anhydrous glucose. The fractionation is better effected when the two sugars are in a crystalline form. The deproteinized and defatted milk filtrates were therefore neutralized, concentrated *in vacuo* and brought to dryness in a vacuum desiccator. The almost crystalline residues were extracted with cold methyl alcohol. No glucose could be identified by means of its osazone in these extracts after evaporation and resolution in small volumes of water.

It has already been mentioned that the diabetic symptoms disappeared for a certain period during lactation and then reappeared, but the results of the above experiments which were performed both in the presence and absence of these symptoms were always the same, thus indicating the ability of the mammary glands to prevent the leakage of blood glucose into the milk of diabetics even in the presence of a high concentration of blood glucose. The synthetic power of these glands appears to afford an efficient mechanism for transforming the whole of the sugar secreted in milk into lactose regardless of the level of the blood sugar. The absence of such a mechanism from other glands may explain the appearance of glucose in their secretions in diabetes.

Carbohydrate metabolism during lactation

Lactose synthesis by the mammary glands from blood sugar might be thought of, in the first instance, as one of the possible explanations of the disappearance of diabetic symptoms for a certain period during lactation. In the latter condition the blood sugar of both normal and diabetic persons finds a new route for its consumption and a normal person will be in a state approaching that of a diabetic in that there is a waste of sugar from the body with resulting polyphagia; in case of the diabetic the removal of sugar in the milk, which would otherwise be lost in the urine, may temporarily remove the diabetic symptoms. On this assumption the only difference between lactating normal and diabetic persons will be that in the former either the glucose stores are mobilized to replace the blood sugar lost in the synthesis of lactose and the glucose loss will be equivalent to the lactose formed or there may be equilibrium between a hyperglycaemia developed during lactation and lactose formation [Bert, 1884; Porcher, 1909, 2]; while in the case of a diabetic person the excess of blood sugar above the normal level is utilized for the lactose synthesis. On this basis the disappearance of diabetic symptoms necessitates the assumption that, during lactation, the whole of the glucose which would otherwise be lost in the urine must be transformed into lactose. In a case of severe diabetes, like case II of the present investigation, the urinary glucose excretion may amount to as much as 500 g. per 24 hr. [Beaumont & Dodds, 1931] on an ordinary mixed diet as was ingested during lactation while, if the milk composition and yield are the same as in a normal person, the lactose produced in 24 hr. would not exceed 51–85 g. Since glucose was not found as such in the milk of diabetics it is clear that the lactose yield should appreciably increase if it was the only factor responsible for the disappearance of the diabetic symptoms during lactation. Lactose occasionally appears in the urine of pregnant and lactating normal individuals, but the urine in the two cases described during the period of absence of diabetic symptoms showed no reduction of Fehling's solution on several occasions and the osazones prepared from these urines resembled the osazone mixture normally present in urine. Winter [1932] proved the absence of lactose from the blood during lactation and of a threshold for this sugar. It follows that there was no replacement of glycosuria by lactosuria in such cases and that any increment in the lactose yield above normal would appear almost entirely in the milk as there could be no retention of this sugar in the blood.

The estimation and comparison of the total lactose yield in the milk of diabetics with that in the milk of normal individuals involve several difficulties. The lactose in mature milk of normal individuals varies from 6 to 8%. It can be seen (Table 2) that the percentage in the milk of the two cases examined during the period of disappearance of diabetic symptoms is within the range for normal individuals. It cannot be said what percentage would be present if these

persons were normal, but the difference between the usual lower and upper limits is only 2% which does not appear to account for the disappearance of glycosuria, especially in a severe case. Still, there is the possibility of an abnormally excessive yield of a milk with a normal percentage of lactose but estimates of milk yields in women are subject to error from many sources.

In view of these difficulties in obtaining an accurate estimate of normal milk yield it was thought that at least some idea of milk yield might be gained from analytical data. On the assumption that in diabetes there is no reason for an increase in the yield of milk constituents other than lactose it is conceivable that if there is an increase of milk yield, in such cases, although the percentage of lactose may remain within the normal range yet the milk will be dilute as regards its other constituents. The fact that glycosuria reappeared after a certain period of lactation allowed a comparison between the percentages of lactose and the constitution of milks obtained during the periods when there was no loss and when there was a loss of sugar in the urine in one and the same person. Further, it would be expected that if the percentages of all the constituents of milk were within the normal range, during either of the two periods, an increase in milk yield would aggravate the condition of the lactating patient, which is contradictory to the actual observation.

For the collection of milk for analysis the instructions of Talbot [1919] were followed. The milk was collected at about 10 a.m.; 20 ml. were drawn both before and after nursing and the two samples were thoroughly mixed. The specific gravity, total solids, lactose, solids other than lactose and ash content were estimated in each case. Table 2 shows the constitution of mature milk during the periods of disappearance and of reappearance of glycosuria, a comparison between the two kinds of milk and between these and the constitution of mature milk of normal individuals.

Table 2

Conditions	Specific gravity average at 15°	Total solids average %	Lactose average %	Solids other than lactose average %	Ash average %
Case I: no glycosuria	1030·7	13·27	6·09	7·18	0·19
Case I: glycosuria	1031·0	13·28	6·10	7·18	0·20
Case II: no glycosuria	1032·6	14·24	7·04	7·20	0·21
Case II: glycosuria	1032·6	14·22	7·04	7·18	0·21
Normal range	1031·0*	11–15·25	6–8	5–7·25	0·18–0·25

* Average at 60° F.

It is clear that the constitution of the milk of lactating diabetics is the same during the disappearance and the reappearance of glycosuria and in both cases it does not differ from the constitution of the milk of normal individuals. The fat content was also estimated in some samples and was always within the normal range. It may be mentioned that the percentages of the constituents in all the individual samples examined did not differ appreciably from the average percentages given in Table 2.

After the reappearance of glycosuria it was arranged to draw the samples of milk at the close of 24 hr. during which the urine was collected. This technique was followed in case II; the samples of urine always showed a strong reduction and gave abundant typical glucosazone crystals. The amount of sugar in the urine varied from 26 to 33 g. per 24 hr. which suggested that the hyperglycaemia which recurred while lactation was still proceeding was of a milder nature than before pregnancy. This is further indicated by the estimations shown in Table 3

which also show that the hyperglycaemia, after the cessation of lactation, returns almost to the high level obtaining before pregnancy.

It might be thought that the disappearance of glycosuria for a certain period during lactation was due to a temporary rise in the renal threshold for glucose, but determination and comparison of the fasting blood sugar levels in case II in the different conditions indicated in Table 3 excluded this idea.

Table 3

Case II in different conditions	Fasting blood sugar level mg. per 100 ml.
Before pregnancy	247
During lactation: no glycosuria	99
During lactation: glycosuria	175
40 days after cessation of lactation	241

It is clear that in this case of severe diabetes the blood sugar level in absence of glycosuria was within the normal limits (90–110 mg. per 100 ml. blood) and that the appearance or absence of glycosuria was quite dependent on the level of the blood sugar.

Basal metabolic rate during lactation

The possibility of the prevalence of factors which raise the metabolic rate during lactation may explain the disappearance of glycosuria in this condition. Fluctuations in oxygen consumption in women are stated to occur; thus the oxygen consumption may fall to 170 ml. per min. during menstruation and rise to 195 ml. 10 days later. Such fluctuations appear to be correlated with variations in the production of sexual hormones which are at a minimum during menstruation and which, according to Verzár & Árvay [1931], increase the basal metabolism in females. Lee [1928] also observed an increase in basal metabolism in rats in dioestrus and pro-oestrus but no change in any other period of the cycle. The disappearance of glycosuria for a certain period during lactation and its reappearance cannot be ascribed to fluctuations in the production of the ovarian hormones since the glycosuria disappeared entirely, in the two cases examined, during the inhibition period of menstruation which follows parturition when it is supposed that there is a deficiency in the production of these hormones, while once the glycosuria starts to reappear during lactation it continues during every period of the menstrual cycle. It follows that if there is any increase in basal metabolism during lactation it will, in all probability, be caused by different factors which operate in this condition. For the comparison of the basal metabolism during lactation with that in the non-lactating condition it was thought advisable to make the comparison under conditions which cover all the cyclic fluctuations in the non-lactating state and, for this purpose, the comparison of the basal metabolism in short-cycle animals such as the rat in which the cycle occurs every 4 or 5 days is more instructive especially when the study is extended for 7 or 8 days.

A number of female rats of the same litter and weight were chosen. Some were allowed to become pregnant and some were left as controls. One week after parturition the oxygen consumption of both the lactating rats and the controls was measured in a closed system. Four pairs of rats of the same weight 160 g. were chosen of each batch. Two pairs of the four of each batch were alternately deprived of food at 4 p.m. on the day preceding the experiments which were performed on consecutive days. On the days of the experiments the oxygen consumption of each pair of the two of each batch was separately

estimated. The consumption in the first 30 min. was ignored in each case in order to avoid the effect of handling and fear of the apparatus. It was found that the oxygen consumption of the lactating rats was within the range for the non-lactating animals which varied from 160 to 175 ml. O_2 per pair per 30 min. during 8 days. It appears, therefore, that there is no recognizable increase in the basal metabolic rate during lactation and that this factor is unlikely to explain the disappearance of glycosuria in lactating diabetics.

Summary

1. In lactating diabetics no glucose is secreted in the milk regardless of the existence or absence of hyperglycaemia. The amounts of lactose and other constituents of the milk remain within the range for normal individuals.

In diabetics, the active mammary glands, contrary to other glands, prevent glucose leakage, possibly by virtue of their synthetic power for lactose, and are capable of maintaining normal milk composition.

2. The diabetic symptoms in the two cases examined entirely disappeared for a certain period during lactation and reappeared while lactation was still proceeding. The hyperglycaemia recurring during lactation was of a milder nature than both before pregnancy and after cessation of lactation.

3. Lactation has no effect on the basal metabolic rate of rats.

4. The disappearance of hyperglycaemia and glycosuria during lactation could not be explained by either (1) secretion of glucose in milk, (2) excessive lactose secretion, (3) replacement of glycosuria by lactosuria or (4) increased sugar combustion.

REFERENCES

Beaumont & Dodds (1931). Recent advances in medicine. London.
Bert (1884). *C.R. Acad. Sci., Paris*, **98**, 775.
Grant (1936). *Biochem. J.* **30**, 2033.
Kaufmann & Hagne (1906). *C.R. Acad. Sci., Paris*, **143**, 779.
Lee (1928). *Amer. J. Physiol.* **86**, 694.
Paton & Cathcart (1911). *J. Physiol.* **42**, 179.
Porcher (1909, 1). *Biochem. Z.* **23**, 370.
—— (1909, 2). *Arch. int. Physiol.* **8**, 356.
Röhmann (1919). *Biochem. Z.* **93**, 237.
Talbot (1919). *J. Amer. med. Ass.* **73**, 662.
Trey (1901). *Z. phys. Chem.* **18**, 193.
Verzár & Árvay (1931). *Biochem. Z.* **240**, 28.
Winter (1932). *J. Physiol.* **77**, 100.

2. THE FIXATION OF CO_2 BY CELL SUSPENSIONS OF *PROPIONIBACTERIUM PENTOSACEUM*[1]

By HARLAND GOFF WOOD and CHESTER HAMLIN WERKMAN

From the Bacteriology Section, Iowa Agricultural Experiment Station, Ames, Iowa, U.S.A.

(*Received 3 November 1939*)

The fixation of CO_2 by heterotrophic non-photosynthetic bacteria was first reported by Wood & Werkman [1936; 1938] in the case of the fermentation of glycerol by the propionic acid bacteria. It was established that the CO_2 utilized and the succinic acid formed are approximately equimolar. These results have been confirmed by Phelps *et al.* [1939]. Wood & Werkman [1938] have suggested that succinic acid is formed in this fermentation by synthesis from a 3-carbon compound and CO_2. The present investigation is a continuation of these studies to determine whether similar considerations apply to the fermentation of other substrates. It is not improbable that the formation of succinic acid by synthesis from CO_2 and a 3-carbon compound may occur more generally than has been suggested.

Methods

Barcroft-Warburg manometric technique was used according to Dixon [1934]. 2 ml. of mixture were used in each cup unless otherwise stated. The temperature of the bath was 30°. The flasks were oscillated continuously 100 times per min. through an amplitude of 5 cm. *Propionibacterium pentosaceum* (49 W) was grown at 30° for 5 days in a medium of yeast extract (Difco) 0·4 %, glycerol 0·5 %, and 0·05 *M* phosphate buffer (*p*H 6·9). The growth was harvested by centrifuging and washed twice with distilled water by suspending 1 g. of wet bacteria in 20 ml. of water. Unless otherwise stated the reaction mixture contained the following concentrations of constituents: substrate 0·2 %, phosphate buffer 0·1 *M* (*p*H 6·15), and 0·025 g. wet bacterial paste per ml. The bacteria, phosphate buffer and other constituents (cf. Tables) were placed in the main chamber of the respirometer flask and the substrate in the side cup. Time of reaction was 18 hr. with the exception of the experiment described in Table 5. The atmosphere was CO_2 with one exception (cf. Table 2).

Under an atmosphere of CO_2 the phosphate buffer takes up much CO_2. During the dissimilation, CO_2 is liberated from the buffer by the acids which are formed. It was therefore necessary to determine the CO_2 bound by the buffer in order to calculate the CO_2 formed or utilized in the dissimilation. This CO_2 was determined manometrically in a mixture containing all constituents except the bacteria, by acidifying with 0·25 ml. of 3 *N* H_2SO_4. Likewise the CO_2 was liberated from the reaction mixture at the conclusion of the dissimilation. The CO_2 produced or utilized in the dissimilation of the substrate is the sum of the CO_2 liberated in the dissimilation and that obtained by acidification at the conclusion of the dissimilation minus the CO_2 originally bound by the buffer.

[1] Journal paper No. J-688 of the Iowa Agricultural Experiment Station. Project No. 572.

The CO_2 formed by the bacteria in the absence of added substrate was usually measured (endogenous CO_2). This value was subtracted from that obtained when substrate was added in all experiments except those of Table 5. Although it is not certain that the endogenous dissimilation proceeds unchanged in the presence of added substrate, nevertheless, endogenous values were subtracted in experiments involving low substrate concentrations (0·2 %) because the gas volumes are small and failure to apply this correction might lead to erroneous conclusions with certain substrates. Since the cells in the absence of added substrate took up CO_2, subtraction of these values makes the apparent CO_2 uptake in the presence of substrate appear smaller; therefore any error does not yield evidence supporting CO_2 utilization.

Certain experiments (Table 2) were conducted in an atmosphere of N_2. In this case the CO_2 was absorbed in 0·3 ml. of 2 *N* carbonate-free NaOH placed in one of the side cups of the flask. At the conclusion of the experiment the alkali was tipped into the main chamber and acidified with 0·4 ml. of 3 *N* H_2SO_4. The liberated CO_2 was measured manometrically.

The procedure in the experiments of Table 5 required modification of technique in that the $NaHCO_3$ buffer contained more bound CO_2 than could be determined at one reading of the manometer. This difficulty was partly overcome by reducing the volume of the reaction mixture to 1 ml. (equivalent to approximately 3600 μl. CO_2 when 1·4 % $NaHCO_2$ was used). During the dissimilation a large part of this CO_2 was liberated so that the remaining bound CO_2 was within the limits of manometric determination.

The original bound CO_2 was determined by acidifying 0·6 ml. (without bacteria) of the 0·72 % $NaHCO_3$ mixture and 0·3 ml. of the 1·4 % $NaHCO_3$ mixture; each was equivalent to approximately 1000 μl. of CO_2. When the manometer was set initially at scale-readings 300 on the closed arm and 0 on the open arm, the 1000 μl. of gas displaced the manometer to 0 on the closed arm and approximately 300 on the open arm, final readings. These readings were adjusted to correspond to those that would be obtained if the measurements had been made at constant volume, using 150 as the zero point, by the following manipulations. The stopcock was opened and the Brodie solution adjusted to 300 in both arms of the manometer. Then the change in reading was determined when the manometer fluid was lowered to 150 in the closed arm. This value was added to the original reading (0) giving the value corresponding to a 150 setting. The correction for the final reading likewise was obtained by setting the manometer fluid at 0 and 0 and determining the change on adjustment to 150. This value was added to the final reading. Calculation has shown that there is a small error in this procedure but the errors of the two operations largely compensate each other.

The concentration of substrate in the original mixture was determined by weight. When reducing sugars were used, the residual sugar was determined by the method of Stiles *et al.* [1926]. Residual polyhydric alcohols were determined by oxidation with HIO_4. The polyhydric alcohol (less than 6 mg. in 10 ml.) plus 2 ml. of 3·6 *N* H_2SO_4 and 25 ml. of 0·0625 *N* HIO_4 was heated for 10 min. on a steam bath. The mixture was cooled and 2 ml. of 50 % KI were added. After 3 min. 25 ml. of water were added and then by pipette 25 ml. of 0·5 *N* $Na_2S_2O_3$ with vigorous shaking. The titration was completed with 0·01 *N* $Na_2S_2O_3$.

(Blank titration − titration of determination) × normality of $Na_2S_2O_3$ × 23·00 = mg. of glycerol. For erythritol the ml. of *N* $Na_2S_2O_3$ are multiplied by 20·33, for adonitol by 19·00 and for mannitol by 18·20.

Experimental

Comparative fixation of CO_2 with various substrates. Glycerol was the only substrate which had been shown by previous investigations to be dissimilated with an uptake of CO_2. Moreover, the action of cell suspensions had not been investigated. Dissimilation of a number of substrates by cell suspensions was therefore undertaken. Table 1 gives representative results from one of a number

Table 1. *Comparative utilization of CO_2 by cell suspensions of* P. pentosaceum *(49 W) with various substrates*

Bacteria with no substrate took up 64 μl. of CO_2

Substrate	Pyruvic acid	Lactic acid	Glyceraldehyde	Glucose	Dihydroxyacetone	Arabinose
μl. CO_2 per 2 ml.	720	322	94	49	48	3

Substrate	Galactose	Xylose	Mannitol	Adonitol	Erythritol	Rhamnose	Glycerol
μl. CO_2 per 2 ml.	−17	−25	−177	−207	−252	−252	−355

of experiments. There was an evolution of CO_2 from the substrates shown in the upper part of the table and an uptake in the case of substrates shown in the lower part. The actual comparative utilization of CO_2 is difficult to determine owing to variation in the relative state of oxidation of the substrate. The following three equations illustrate this point:

(O.R. = 1). $3CH_3COCOOH + H_2O = CH_3CH_2COOH + 2CH_3COOH + 2CO_2$.

(O.R. = 0). $3CH_3CHOHCOOH = 2CH_3CH_2COOH + CH_3COOH + CO_2 + H_2O$.

(O.R. = −1). $CH_2OHCHOHCH_2OH = CH_3CH_2COOH + H_2O$.

If pyruvic acid which has a redox (O.R.) value of 1, is dissimilated to propionic acid, acetic acid and CO_2, one obtains 2 mol. of CO_2 for each 3 of pyruvic acid. In the case of lactic acid with an O.R. value of 0, 1 mol. of CO_2 is obtained from each 3 of lactic acid. Glycerol has a negative O.R. value and a balanced equation is obtained in this case in which no CO_2 is formed. Obviously, if CO_2 is produced in one reaction of the dissimilation and utilized in another, utilization will be apparent only if the uptake is greater than the production. In the case of pyruvic and lactic acids, the observed CO_2 formation was in approximate agreement with that calculated from the equations and probably little CO_2 was utilized. Glyceraldehyde, glucose, dihydroxyacetone and galactose are either 3- or 6-carbon compounds of the same oxidation-reduction level as lactic acid. A quantity of CO_2 equivalent to that from lactic acid is to be expected in these cases if there is no fixation of CO_2 and the dissimilation of the compounds is complete; the decrease in the rates of CO_2 evolution indicated that the substrates were completely fermented. Consequently the observed decrease in the production of CO_2 as compared with lactic acid (Table 1) may be an approximation of the amount of fixation of CO_2 occurring in the dissimilation of these substrates. Of course, conversions other than those expressed in the equation for lactic acid dissimilation (apart from CO_2 utilization) may take place, but qualitatively the above conclusions are undoubtedly true. In the case of the reduced compounds mannitol, adonitol, erythritol, rhamnose and glycerol, the formation of CO_2 is less than the utilization and the net result is the observed CO_2 uptake. These results prove that there is fixation in the dissimilation of a number of compounds (those in the lower part of Table 1) and indicate that CO_2 may have been utilized with the other substrates although an actual CO_2 uptake could not be demonstrated.

Comparative utilization of CO_2 under CO_2 and N_2. The utilization of CO_2 might be expected to increase with concentration of CO_2, and in fact this is shown to be true with a number of substrates (Table 2). CO_2 in the fermentation under N_2 was absorbed in alkali and thus was continuously removed from the medium, whereas in the comparative experiment the medium was saturated with CO_2. A survey of the results in Table 2 shows with each substrate that the formation of CO_2 was less or utilization was greater with an atmosphere of CO_2 than with N_2. The CO_2 values are expressed as mM per 100 mM of substrate and therefore are on a comparable basis. Differences between the CO_2 values under N_2 and under CO_2 probably represent roughly the change in CO_2 utilization under the two conditions. These experiments offer additional evidence that CO_2 is utilized in the dissimilation of a number of substrates with which a direct CO_2 uptake could not be demonstrated. It is possible that these changes in CO_2 values are caused by some change in the mechanism of dissimilation other than CO_2 utilization but this explanation is not supported by present evidence.

Inhibition of fixation of CO_2. In order to obtain information which might be more readily applicable to the study of tissues, a number of physiologically active inhibitors were tested for their effects on CO_2 utilization in the dissimilation of glycerol (Table 3). Fluoride, iodoacetate, malonate, azide, arsenite, cyanide and pyrophosphate were tested. Malonate and pyrophosphate were used especially because they inhibit succinic dehydrogenase and arsenite because of its effect on decarboxylation of β-keto-acids. A possible mechanism accounting for CO_2 utilization as well as succinic acid formation involves the addition of CO_2 to pyruvic acid to form oxaloacetic acid, followed by reduction to malic acid, dehydration to fumaric acid and finally reduction to succinic acid. Neither malonate nor pyrophosphate affected utilization of CO_2. The values obtained were 29·7 mM and 23·9 mM compared with 29·8 mM with no addition of inhibitor. It is possible that the process was stopped at fumaric acid. Arsenite might be expected to prevent the formation of oxaloacetic acid. However, this does not seem probable, for 0·04 M arsenite did not inhibit the breakdown of pyruvic acid by propionic acid bacteria. These points will be considered in a subsequent report.

It is of interest that cyanide and azide inhibit photosynthesis but are without effect on fixation of CO_2 by the propionic acid bacteria. This observation might lead one to suggest that the two processes do not involve similar reactions. However, the fixation of CO_2 in photosynthesis is closely associated with respiration. It is possible that the same process occurs in the fixation of CO_2 in the dark by propionic acid bacteria as in photosynthesis and the cyanide and azide inhibit photosynthesis by their action on the respiratory system.

Only NaF and iodoacetate of the group of inhibitors tested inhibited CO_2 utilization. Iodoacetate was not very suitable, however, because it likewise suppressed the entire dissimilation when used in effective concentrations, only 36·7 % of the glycerol being fermented. NaF is quite satisfactory. When no NaF was added, 29·8 mM of CO_2 were utilized per 100 mM of glycerol. A concentration of 0·000625 M NaF reduced the utilization of CO_2 to 8·8 mM and with 0·00125 M NaF utilization was prevented (1·5 mM) although the dissimilation was by no means completely inhibited. Table 4 shows the comparative effects of fluoride on the utilization of CO_2 with a number of substrates. In each dissimilation utilization of CO_2 was apparently inhibited although galactose and arabinose were not greatly affected probably because there was little utilization of CO_2. In similar experiments (Table 2) in which CO_2 utilization probably occurred, only 15·8 and 19·7 mM of CO_2 were formed from galactose and

Table 2. *Comparative formation of CO_2 from various substrates by cell suspensions of* P. pentosaceum *(49 W) under CO_2 and under N_2*

Bacteria with no substrate took up 32 µl. of CO_2 under CO_2 and produced 13 µl. under N_2

Substrate	Glucose		Galactose		Arabinose		Dihydroxy-acetone		Mannitol		Adonitol		Glycerol	
Atmosphere	N_2	CO_2	N_2	CO_2	N_2	CO_2	N_2	CO_2	N_2	CO_2	N_2	CO_2	N_2	CO_2
% substrate fermented	97·5	96·1	73·5	67·1	55·4	49·2	99·3	97·3	31·0	32·7	83·0	81·0	93·0	94·2
µl. CO_2 per 2 ml.	177	106	83·5	26·5	74·5	29	97·0	77·5	33	−22	0·0	−114	0·0	−216
m*M* CO_2 per 100 of fermented substrate	36·5	21·8	45·7	15·8	45·1	19·7	39·2	21·5	21·5	−13·6	0·0	− 23·8	0·0	− 23·5

Table 3. *Effect of inhibitors on utilization of CO_2*

Inhibitor	None	NaF		Iodoacetate		Malonate	NaN_3	$NaAsO_2$	KCN	Pyro-phos-phate
Molarity of inhibitor	—	0·00125	0·000625	0·000312	0·000078	0·03	0·002	0·01	0·1	0·02
% glycerol fermented	90·0	62·0	90·2	36·7	48·5	81·5	94·2	93·7	80·1	85·2
µl. CO_2 per 2 ml.	−261	− 9	−77	−35	−82	−235	−216	−280	−172	−198
m*M* CO_2 utilized per 100 of fermented glycerol	29·8	1·5	8·8	9·8	17·3	29·7	23·5	30·7	22·1	23·9

Table 4. *Effect of NaF on the utilization of CO_2 from various substrates*

Substrate	Glycerol		Adonitol		Mannitol		Glucose		Galactose		Arabinose	
Molarity of NaF	None	0·001	None	0·001	None	0·001	None	0·001	None	0·00125	None	0·001
% substrate fermented	99·3	90·8	94·3	97·0	94·3	48·6	99·3	90·1	98·6	92·7	79·1	69·1
µl. CO_2 per 2 ml.	−241	−20	−152	117	46	89	55	151	316	364	191	178
m*M* CO_2 per 100 of fermented substrate	− 22·3	− 2·3	27·3	20·5	9·9	37·2	22·2	67·5	64·3	78·8	40·4	43·1

arabinose as compared with 67·5 and 40·4 in the present experiment. In the case of glucose with no NaF present, 22·2 mM of CO_2 were formed. When CO_2 utilization was inhibited by NaF, the production of CO_2 increased to 67·5 mM. Assuming that the observed CO_2 is the net result of both formation and utilization of CO_2, this increase is to be expected since uptake of CO_2 was eliminated. Although the only infallible proof of CO_2 utilization lies in a direct demonstration of CO_2 uptake, the increased production of CO_2 in the presence of a known inhibitor is strong evidence of utilization, even in those dissimilations not affording direct proof.

In preparation for quantitative investigations involving determination of all products of dissimilation, a stronger buffer was used so that more substrate would be fermented. The results (Table 5) show the effect of increasing concentration of NaF on the utilization of CO_2 in the dissimilation of glucose (upper part of table) and glycerol (lower part of table) with 0·72 % $NaHCO_3$ and 1·6 %

Table 5. *Effect of increasing concentration of NaF on the utilization of CO_2 in glucose and glycerol dissimilations*

0·05 g. wet bacteria per ml.

NaF M	Substrate fermented %	CO_2 per ml. μl.	CO_2 per 100 mM of substrate mM
		Time = 20 hr.	
	0·72 % $NaHCO_3$		0·8 % glucose
0·000	100·0	287	30·0
0·001	99·6	349	36·6
0·005	97·3	352	37·8
0·0075	93·1	387	43·4
0·010	79·0	426	56·3
0·015	42·5	—	—
0·020	15·6	—	—
		Time = 30 hr.	
	1·44 % $NaHCO_3$		1·6 % glucose
0·000	99·8	556	28·0
0·001	99·5	586	29·6
0·0025	98·9	821	41·7
0·005	96·8	884	45·9
0·010	95·2	998	52·6
0·015	78·1	1034	66·5
0·020	65·9	812	61·9
		Time = 33·5 hr.	
	0·72 % $NaHCO_3$		0·8 % glycerol
0·000	95·7	− 647	− 34·5
0·005	75·7	− 263	− 17·7
0·0075	69·9	− 219	− 16·0
0·010	62·8	− 66	− 5·4
0·015	44·3	+ 24	2·8
		Time = 45·5 hr.	
	1·44 % $NaHCO_3$		1·6 % glycerol
0·000	78·7	− 1594	− 52·0
0·005	77·5	− 1468	− 48·6
0·0075	75·2	− 1254	− 42·8
0·010	69·8	− 941	− 34·6
0·015	41·5	− 284	− 17·5

substrate. The inhibition of fixation of CO_2 by increasing concentrations of NaF is clearly shown in the case of glycerol. The fixation decreased from 34·5 mM to a production of 2·5 in the one case and from 52·0 mM to 17·5 in the other case with increase in concentration of NaF. With glucose, as fixation of CO_2 was inhibited, the yield of CO_2 increased from 30·0 to 56·3 and from 28·0

to 61·9 mM. However, the effective molarity is much higher in $NaHCO_3$ buffer than in phosphate buffer. With 0·72 % $NaHCO_3$ *c.* 0·010 M NaF was necessary effectively to stop CO_2 utilization, whereas with 1·4 % $NaHCO_3$, 0·015 M NaF was required. This concentration of NaF is approximately ten times that required to inhibit utilization of CO_2 with phosphate buffer (Table 3). The *p*H values of the respective buffers after saturation with CO_2 were: (1) phosphate buffer 0·1 M (21 ml. 0·1 M K_2HPO_4 and 79 ml. 0·1 M KH_2PO_4), *p*H 5·8; (2) 0·72 % $NaHCO_3$, *p*H 6·6; (3) 1·4 % $NaHCO_3$, *p*H 6·8.

Lipmann [1929] has shown that inhibition of glycolysis by NaF is a function of *p*H. Apparently the same phenomenon occurs in inhibition of fixation of CO_2 by NaF.

The fact that both utilization of CO_2 and fermentation of phosphoglycerate are inhibited by NaF suggests a relationship of the two processes. In this case the effective concentration of NaF should be identical in the two processes. Table 6 shows the effect of increasing concentration of NaF on the dissimilation

Table 6. *Effect of increasing concentration of NaF on the dissimilation of phosphoglycerate*

Molarity of NaF	0·0000	0·0004	0·0007	0·0015	0·0050	0·0100
μl. CO_2 per 2 ml. from phosphoglycerate	341	312	306	184	86	91
μl. CO_2 per 2 ml. (endogenous)	59	66	66	59	76	90
Difference	282	246	240	125	10	1

Reaction mixture contained 0·225 % phosphoglyceric acid (adjusted to *p*H 6·9), 0·72 % $NaHCO_3$, 0·050 g. wet bacteria per ml. and varied concentration of NaF. Atmosphere CO_2. Reaction time 18 hr. Bacteria were harvested from 6 days' growth in medium containing glucose 0·5 %, $NaHCO_3$ 1·0 % and yeast extract (Difco) 0·4 %.

of phosphoglycerate on the basis of CO_2 evolution. A molarity of 0·005 almost completely stopped the dissimilation of phosphoglyceric acid. This concentration of NaF only partially stopped utilization of CO_2 and 0·01 M NaF was necessary for substantially complete inhibition (Table 5). These results leave some question as to whether the same reaction is being affected in the two processes. The nature of the experiments precludes an exact duplication of experimental conditions and the observed differences in the concentrations of NaF may be caused by some uncontrolled factor. A greater concentration of NaF is required to inhibit the dissimilation of phosphopyruvic acid than phosphoglyceric acid by muscle (0·01 M inhibits phosphoglyceric acid 100 % and phosphopyruvic acid 20 % [Lohman & Meyerhof, 1934]). Possibly CO_2 utilization involves the latter compound. The effect of iodoacetate on utilization of CO_2 may be due to the inhibition of pyruvic acid breakdown. The activity of NaF indicates that utilization of CO_2 has a close connexion with phosphorylation. Further evidence for this suggestion is that phosphate is necessary for optimal CO_2 utilization and succinic acid formation [cf. Wood & Werkman, 1940]. From the standpoint of thermodynamics this concept seems reasonable since the high energy content of the phosphorylated compounds may be the source of energy for CO_2 uptake.

Summary

The fixation of CO_2 by cell suspensions of *Propionibacterium pentosaceum* has been investigated using a variety of substrates. The dissimilation of mannitol, adonitol, erythritol, glycerol and rhamnose under an atmosphere of CO_2 was accompanied by a definite uptake of CO_2, whereas glyceraldehyde, glucose,

dihydroxyacetone, arabinose, galactose and xylose gave a small evolution or some uptake. The evolution of CO_2 from lactic and pyruvic acids was much larger. Dissimilation of most of the substrates in an atmosphere of N_2 occurred with an evolution of CO_2 which was usually much larger than in an atmosphere of CO_2, indicating that utilization of CO_2 is proportional to the CO_2 concentration. These results suggest that the fixation of CO_2 may be a phenomenon of widespread occurrence.

Malonate, azide, arsenite, cyanide and pyrophosphate had no influence on CO_2 fixation. NaF and iodoacetate inhibit CO_2 utilization but iodoacetate is not satisfactory because it suppresses the entire dissimilation. NaF increased the evolution of CO_2 from a number of substrates. This fact is further evidence of its utilization in their dissimilation. The concentration of NaF necessary to inhibit dissimilation of phosphoglyceric acid was about one-half that required to inhibit utilization of CO_2. The reactions inhibited may not be identical in the two processes. It is probable, however, that a phosphate ester, possibly phosphopyruvic acid, is involved in the utilization of CO_2.

REFERENCES

Dixon (1934). Manometric methods. Camb. Univ. Press.
Lipmann (1929). *Biochem. Z.* **206**, 171.
Lohman & Meyerhof (1934). *Biochem. Z.* **273**, 60.
Phelps, Johnson & Peterson (1939). *Biochem. J.* **33**, 726.
Stiles, Peterson & Fred (1926). *J. Bact.* **12**, 427.
Wood & Werkman (1936). *Biochem. J.* **30**, 48.
—— —— (1938). *Biochem. J.* **32**, 1262.
—— —— (1940). *Biochem. J.* (to be submitted).

3. THE EFFECT OF SOME SATURATED FATTY ACIDS ON THE RESPIRATION OF BAKER'S YEAST

By ELTON S. COOK and Sister MARY NORBERT MORGAN, R.S.M.
From the Institutum Divi Thomae, Cincinnati, Ohio

(*Received 27 November 1939*)

In the course of a study [Cook *et al.* 1938, 1] of the effects of certain fractions from yeast on the respiration of yeast and tissues it was observed [Cook & Kreke, 1938, 1] that a water-insoluble steam distillate obtained from an aqueous-alcoholic extract of yeast caused a marked increase in O_2 uptake when added to a yeast suspension but depressed the respiration of rat skin [Cook & Kreke, 1938, 2]. From its properties it soon became evident that the bulk of the distillate was composed of saturated fatty acids, and preliminary experiments showed similar respiratory activities to be possessed by decoic and undecoic acids [Cook & Kreke, 1938, 2; 1939].

It is well known that many animal tissues, such as liver, kidney and spleen, effect the oxidation of fatty acids *in vitro* [Quastel & Wheatley, 1933; 1934; Jowett & Quastel, 1935; Quagliarello, 1932, 1, 2; Quagliarello & Scoz, 1933]. Fatty acids inhibit the oxidation of pyruvate by brain, apparently by displacement of one of the members of the pyruvate-oxidase system [Peters & Wakelin, 1938], and also inhibit the respiration of rat skin [Cook & Kreke, 1938, 2; 1939]. Ciaranfi [1938] has shown that tumour tissue is unable to oxidize saturated and unsaturated fatty acids *in vitro* and that some acids inhibit tumour respiration, a fact which has been confirmed in our laboratory. Less has been written concerning the oxidation of fatty acids by yeast. It has been well established, particularly by the work of Smedley-Maclean [Smedley-Maclean & Hoffert, 1923; 1926; Macleod & Smedley-Maclean, 1938] and of Wieland & Wille [1935], that yeast, when incubated in oxygenated solutions, converts acetate into fat, at the same time burning part of it, but formate, propionate or butyrate do not seem to influence the production of fat [Smedley-Maclean & Hoffert, 1923]. Wieland & Sonderhoff [1932] have found that yeast oxidizes acetate to CO_2 and water and also in part to succinic and citric acids under the proper conditions. Winzler & Baumberger [1938] report that yeast oxidizes 58·7% of Na acetate to CO_2 and water and assimilates the remainder.

The purpose of this paper is to show the relative effects of a series of fatty acids on the O_2 uptake of baker's yeast and of certain of these acids on the R.Q. The facts observed are consonant with the oxidation of these acids by yeast, but proof of this must await a detailed chemical study of the fate of the acids.

Experimental

Fatty acids. The saturated fatty acids employed were Eastman Kodak products and were used without further purification. A specially purified sample of sodium ricinoleate was also tested and was supplied through the courtesy of

Dr R. S. Shelton of the Wm S. Merrell Co., Cincinnati, Ohio. Stock solutions of the fatty acids containing about 50 mg. per ml. were prepared and adjusted to *p*H 7·3 (glass electrode) with NaOH. This brought some of the lower acids into solution and partially dissolved the others with the formation of a uniform suspension. Warming to 37·5° increased the solubility. It is evident that at this *p*H mixtures of acid and Na salt existed [cf. Peters, 1931].

Respiration technique. The direct Warburg manometric technique was employed with slight changes from that described in previous papers [Cook *et al.* 1938, 1, 2; Cook & Kreke, 1939; Norris & Kreke, 1937]. Fleischmann's baker's yeast from the centre of a fresh 1 lb. cake was washed 3 times by centrifuging with the suspending medium and then was made up with fresh medium to a count of 250 as determined by a photoelectric densitometer (count of 1 = 250,000 cells per ml.). Ringer-phosphate-glucose solution (0·02 % glucose, *p*H 7·3) was used as the suspending medium in the earlier experiments.

1 ml. of the yeast suspension (containing an average of 3·9 mg. dry weight of yeast), the desired amount of the acid solution and sufficient Ringer-phosphate-glucose to bring the volume to 3·1 ml. were placed in the manometer flask, and 0·2 ml. *N* KOH was placed in the inner well. After an equilibrium period of 15 min. the manometers were shaken at 120 oscillations per minute for a 25 min. respiration period, in air, at 37·5°. The entire water bath was enclosed in a box maintained at approximately 27°.

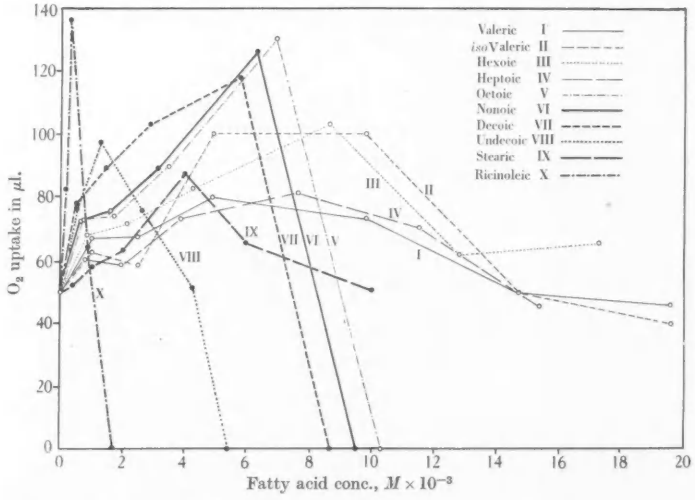


Fig. 1. Effect of added fatty acids on O_2 uptake of baker's yeast in Ringer-phosphate-glucose (0·02 %) at *p*H 7·3 and 37·5° for 25 min. respiration period.

The effects of ten fatty acids on the respiration of yeast under these conditions are shown in Fig. 1. The fatty acids were not oxidized to a measurable extent in the absence of yeast. For the sake of readier comparison all of the results have been calculated to a control respiration of 50 µl. The actual average control respiration (respiration of yeast without added acid) was 48 µl. (range,

40–58 μl.) which corresponds to an average $Q_{O_2}^{air}$ of about 30 with a range of 25 to 36. It will be observed that the acids have optimum effective concentrations. Parallel staining experiments with methylene blue showed that inhibiting concentrations of the acids were toxic to the yeast cells. It has been shown that pyruvic acid, which is converted into fat by yeast, is also toxic to yeast in higher concentrations [Haehn & Glaubitz, 1927; Smythe, 1938]. An optimum concentration of fatty acids also exists for the respiration of liver slices, higher concentrations being inhibitory [Quastel & Wheatley, 1933; 1934; Jowett & Quastel, 1935].

It will be noticed that, in general, there is an increase in physiological activity of the acids with increase in length of the straight carbon chain over the range C_5–C_{11} with the apparent exception of heptoic acid. This is evident both in the increased toxicity and the increased respiratory activity of lower concentrations. Stearic acid, on the other hand, shows a lowered activity. Ricinoleic acid, of interest because of its bacterial detoxifying properties, was found to be very toxic to yeast but very active in non-toxic concentrations. It is of interest that Beck [1935] has found the lower aliphatic acids (acetic to hexoic) to inhibit cytochrome reduction in yeast, and increase in inhibiting power accompanies an increase in length of carbon chain in acid concentrations of 0·01 M. Very little effect was observed in 0·001 M concentration. The addition of Na salts of the acids, however, in part overcomes the inhibitory effects of the free acids. These observations are suggestive in connexion with an ultimate consideration of the mechanism involved. It is to be remembered that the physical properties of the acids, e.g. the amount in solution, their surface activity and the ease of penetration into the cell, will vary with the size of the acid and must be taken into consideration in a final evaluation of the effects. It is, however, not within the province of the present paper to discuss cell penetration although it may be pointed out that the lower fatty acids and their salts rapidly penetrate yeast cells [Beck, 1935]. For discussion of cell penetration by these substances see, for example, Beck [1935], Harvey [1914], Crozier [1916], Smith & Clowes [1924], Smith [1925], Lillie [1926], and Howard [1931]. See also the suggestive film penetration studies of Schulman & Rideal [1937] and Schulman [1937].

Respiratory quotients. In order to investigate the above effects a little further, the influence of hexoic and decoic acids on the R.Q. was studied. It has been shown [Stier & Stannard, 1936; Warburg, 1927] that baker's yeast in a phosphate medium free of glucose oxidizes endogenous carbohydrates alone and that the CO_2 produced is purely respiratory uncomplicated by fermentative CO_2. Under these conditions as well as in the presence of 0·02 % glucose we have found the addition of the fatty acids to lower the R.Q.

R.Q. were obtained by the direct two-vessel method [Dixon, 1934]. To obtain the conditions of Stier & Stannard and in order to avoid retention of CO_2 the suspending medium was changed from Ringer-phosphate to M/15 KH_2PO_4 at pH 4·5, as suggested by Krebs [1928]. This made the addition of acid at the end of the experiment unnecessary, as shown by trial, and made it possible to construct time curves. In a number of experiments 0·02 % glucose was added to the medium and, although the total O_2 consumption was raised (Figs. 2 and 3), this amount of glucose did not alter the R.Q. within the limits of experimental error. The acids proved to be more toxic to yeast in the KH_2PO_4 than in Ringer-phosphate solutions and therefore less could be added. It should also be noted that free acids and not Na salts were present in suspension. Their essential behaviour, however, did not appear to be altered since cell penetration was evidently accomplished.

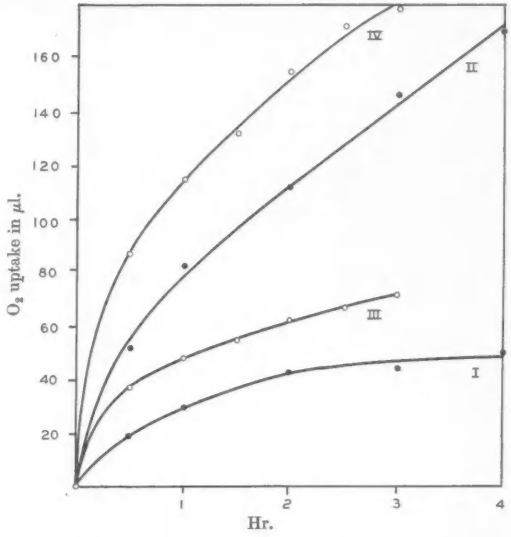


Fig. 2. Effect of $5{\cdot}8 \times 10^{-4}$ *M* decoic acid (0·1 mg. per ml.) on O_2 uptake of baker's yeast in *M*/15 KH_2PO_4 at *p*H 4·5 and 37·5°. Curve I, no acid; curve II, corresponding test with acid; curve III, no acid; curve IV, corresponding test with acid.

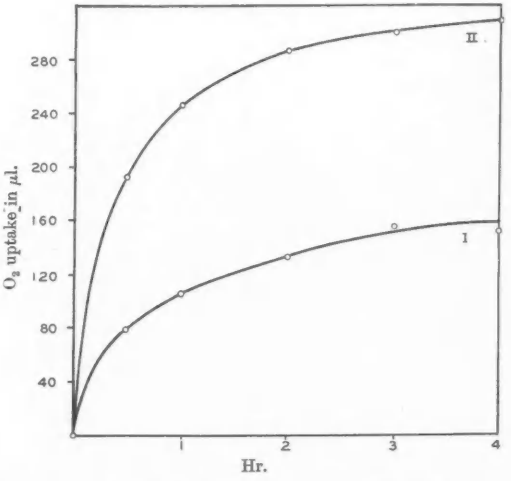


Fig. 3. Effect of $5{\cdot}8 \times 10^{-4}$ *M* decoic acid (0·1 mg. per ml.) on O_2 uptake of baker's yeast in *M*/15 KH_2PO_4 containing 0·02% glucose at *p*H 4·5 and 37·5°. Curve I, no acid; curve II, acid added.

A summary of the results is given in Table 1. It will be seen that the R.Q. of 0·94 in the absence of fatty acids was lowered to 0·80 and 0·73 by $5{\cdot}8 \times 10^{-4}$ *M* decoic acid (0·1 mg. per ml.) and $4{\cdot}3 \times 10^{-3}$ *M* hexoic acid (0·5 mg. per ml.),

Table 1. *R.Q. of baker's yeast in M/15 KH_2PO_4 with and without added fatty acids at pH 4·5 and 37·5°*

Acid	Glucose (0·02%)	R.Q. Average	R.Q. Limits	No. of determinations
—	–	0·94 ±0·03	0·90–1·01	10
—	+	0·93 ±0·02	0·90–0·97	17
Decoic $5{\cdot}8 \times 10^{-4}$ *M*	–	0·80 ±0·03	0·75–0·84	13
,,	+	0·82 ±0·01	0·80–0·84	8
Hexoic $4{\cdot}3 \times 10^{-3}$ *M*	+	0·73 ±0·02	0·68–0·76	8

respectively. The R.Q. in the absence of acid approaches 1 by these measurements and indicates that carbohydrate is being burnt, as pointed out above. The respirations were measured over periods of 3–4 hr. (Figs. 2 and 3) and the R.Q. remained constant during these periods.

Discussion

It is seen that non-toxic concentrations of the fatty acids increase the O_2 uptake of suspensions of baker's yeast. The acids studied depress the R.Q. in the absence of glucose or in the presence of a very low concentration of glucose. These facts suggest the oxidation of the acids by the yeast. Some further evidence is in accord with this suggestion. The flasks to which the acids were added usually gave slight positive tests for ketone bodies by the nitroprusside and *o*-nitrobenzaldehyde reactions. On the other hand, under the conditions of the experiments, insufficient β-keto-acids (acetoacetic acid) were present to be determined manometrically by the procedure of Edson [1935]. Since the amounts of ketone present were near the limits of sensitivity of the colour tests, some of the yeast suspensions were distilled. The first fractions gave definite positive reactions indicating the presence of volatile ketones. The addition of a saturated solution of 2:4-dinitrophenylhydrazine in 2*N* HCl to a few ml. of the distillate gave a very small amount of a yellow crystalline precipitate in the case of the yeast suspensions which had been treated with the fatty acids. Insufficient precipitate was obtained for M.P. determination but microscopically the crystals appeared to be identical with those of acetone-2:4-dinitrophenylhydrazone. These observations are suggestive but not conclusive. A thorough chemical study of the fate of the fatty acids must be carried out to explain the observed effects on yeast respiration. Since circumstances do not permit us to undertake this work at this time we feel it worth while to present our findings to date.

Summary

The addition of non-toxic concentrations of saturated fatty acids to suspensions of baker's yeast causes an increase in O_2 uptake. The magnitude of the effect depends both upon the concentration of the acid and its mol. wt. Within limits (valeric to undecoic) the physiological effects, as determined by toxicity and respiratory activity, generally appear to increase with lengthening of the carbon chain but a lowering of activity is evident with stearic acid.

Measurements of the R.Q. and qualitative tests for the presence of ketone bodies suggest that the respiratory activity may be due at least in part to the oxidation of the fatty acids by the yeast.

REFERENCES

Beck (1935). *Biochem. J.* **29**, 2424.
Ciaranfi (1938). *Amer. J. Cancer*, **32**, 561.
Cook, Hart & Joly (1938, 2). *Proc. Soc. exp. Biol., N.Y.*, **38**, 169.
—— & Kreke (1938, 1). *Studies Inst. Divi Thomae*, **2**, 47.
—— —— (1938, 2). *Nature, Lond.*, **142**, 719.
—— —— (1939). *Studies Inst. Divi Thomae*, **2**, 215.
—— —— & Nutini (1938, 1). *Studies Inst. Divi Thomae*, **2**, 23.
Crozier (1916). *J. biol. Chem.* **24**, 255.
Dixon (1934). Manometric Methods. Cambridge.
Edson (1935). *Biochem. J.* **29**, 2082.
Haehn & Glaubitz (1927). *Hoppe-Seyl. Z.* **168**, 233.
Harvey (1914). *Science*, **39**, 947.
Howard (1931). *Biol. Bull. Wood's Hole*, **60**, 132.
Jowett & Quastel (1935). *Biochem. J.* **29**, 2143, 2159, 2181.
Krebs (1928). In Peterfi, Methodik der wissenschaftlichen Biologie, **2**, 1048. Berlin.
Lillie (1926). *J. gen. Physiol.* **8**, 339.
Macleod & Smedley-Maclean (1938). *Biochem. J.* **32**, 1571.
Norris & Kreke (1937). *Studies Inst. Divi Thomae*, **1**, 137.
Peters (1931). *Proc. roy. Soc.* A, **133**, 140.
—— & Wakelin (1938). *Biochem. J.* **32**, 2290.
Quagliarello (1932, 1). *R. C. Accad. Lincei*, **16**, 387.
—— (1932, 2). *Mem. Accad. Lincei*, **4**, Estratto No. 3, 17.
—— & Scoz (1933). *Arch. ital. Biol.* **90**, 9.
Quastel & Wheatley (1933). *Biochem. J.* **27**, 1753.
—— —— (1934). *Biochem. J.* **28**, 1014.
Schulman (1937). *Trans. Faraday Soc.* **33**, 1116.
—— & Rideal (1937). *Proc. roy. Soc.* B, **122**, 29, 46.
Smedley-Maclean & Hoffert (1923). *Biochem. J.* **17**, 720.
—— —— (1926). *Biochem. J.* **20**, 343.
Smith (1925). *Amer. J. Physiol.* **72**, 347.
—— & Clowes (1924). *Amer. J. Physiol.* **68**, 183.
Smythe (1938). *J. biol. Chem.* **125**, 635.
Stier & Stannard (1936). *J. gen. Physiol.* **19**, 461.
Warburg (1927). *Biochem. Z.* **189**, 354.
Wieland & Sonderhoff (1932). *Liebigs Ann.* **499**, 213.
—— & Wille (1935). *Liebigs Ann.* **515**, 260.
Winzler & Baumberger (1938). *J. cell. comp. Physiol.* **12**, 183

4. OBSERVATIONS ON THE *IN VITRO* SYNTHESIS OF ADRENALINE UNDER PHYSIOLOGICAL CONDITIONS

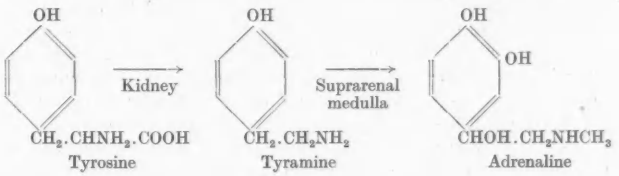
By JOHN DEVINE

From the Department of Physiology, the University, Manchester

(*Received 6 November 1939*)

Raper and his colleagues [Dulière & Raper, 1930; Heard & Raper, 1933] showed that pressor substances having the characteristic properties of adrenaline and adrenalone are produced during the *in vitro* oxidation of certain *N*-methyl derivatives of tyrosine by appropriate chemical reagents or by means of the enzyme tyrosinase. This constitutes the first piece of sound experimental evidence in favour of the theory that tyrosine is the precursor of adrenaline in the animal body. The intermediate reactions are formulated along lines already made familiar by the study of the tyrosine-tyrosinase reaction in the formation of melanin [Raper, 1927]. The actual *in vivo* production of adrenaline in place of the melanin, which appears only pathologically, is considered to be due to the operation of an oxidation-reduction system, or possibly to the limitations imposed on the enzyme reaction by complete substitution of the amino-N in the substrate molecule.

More recently, further progress in the study of this problem has been announced by Schuler & Wiedemann [1935] who claim to have demonstrated for the first time the *in vitro* production of adrenaline or an "adrenaline-like substance" from tyramine. According to their scheme, outlined below, tyramine is first produced in the kidneys by decarboxylation of tyrosine [Schuler *et al.* 1936] and is ultimately converted into adrenaline in the suprarenal medulla.



The evidence for the second stage of this process rests on experiments in which slices of surviving guinea-pig adrenal were incubated at 38° in oxygenated physiological saline or phosphate buffer after various tyrosine derivatives had been added as substrates. Of these, only tyramine caused a substantial increase in the adrenaline content of the solution, as measured by the colorimetric test of Folin and by the increase of pressor activity.

Considering the somewhat unexpected result arrived at, and the apparent finality of this type of experiment as compared with the less direct mode of attack employed by Raper, the claim of Schuler & Wiedemann seems to have been accepted as generally valid in the recent literature without exciting either criticism or independent confirmation. The unreliability of the Folin colour

reaction in this respect has, however, been commented on [Devine, 1937]. The present communication describes an attempt to effect the transformation described by Schuler & Wiedemann in bullock adrenal tissue instead of that of the guinea-pig, and to follow the course of the experiments colorimetrically by means of the more reliable Vulpian reaction (as modified by Schild [1933]) in conjunction with assays of the pressor activity of the solutions.

Attention may be directed at this point to the surprising lack of information as to whether suprarenal tissue itself contains any substance identifiable as an intermediate compound in the hypothetical series of reactions by which tyrosine might be converted into adrenaline. It has long been known that adrenal extracts contain an as yet unidentified catechol derivative[1] which is responsible for some of the discrepancies recorded in earlier colorimetric assays of the adrenaline content. In an examination previously made of its distribution in bullock adrenal gland [Devine, 1936], it was suggested that this substance may be the immediate precursor of adrenaline stored in the adrenal tissue for the final stage of the conversion. As its isolation free from adrenaline has not yet been effected, it was considered of interest to test its behaviour towards surviving adrenal tissue by using suprarenal extracts of known composition as regards the proportion of adrenaline and catechol X.

In all the experiments the original plan was first to eliminate substances showing little or no response to the action of the living tissue by means of the colorimetric test and then to submit only the remainder to a re-examination by physiological assay. The appointment of the writer elsewhere unfortunately necessitated the curtailment of this scheme, which was limited almost entirely to colorimetric assays.

Experimental

1. *Experiments on suprarenal extracts containing catechol X*

Preparation of extracts. As the isolation of catechol X free from adrenaline has not yet been achieved, extracts containing approximately equal portions of both compounds were prepared as follows for each experiment. 400 g. minced bullock adrenal gland were extracted with 6% trichloroacetic acid, and 240 ml. 10% Pb acetate were added to the protein-free filtrate. NH_3 was then added to *p*H 4 and the resulting precipitate washed with water and treated with a slight excess of dilute H_2SO_4. After removal of $PbSO_4$ by centrifuging, the adrenaline and catechol X in the solution ("solution X") were assayed colorimetrically (see footnote below for methods) and the composition adjusted immediately before use to that of Ringer-glucose solution (0·1% glucose) at *p*H 6·5.

Tissue slice experiments. Bullock adrenals were obtained from the abattoir almost immediately after slaughtering and brought in ice to the laboratory (15 min.) to be defatted and sliced. During dissection the slices of medulla or cortex were kept in Ringer solution and then dried on filter paper and weighed out in portions of 80 mg. These portions were then transferred to a series of test tubes containing 5 ml. solution made up of various proportions of Ringer solution and solution X. The tubes were then kept in a thermostat at 38° for 2–2¼ hr. while O_2 from a cylinder was bubbled through slowly and as uniformly as possible. Control experiments in which the slices were suspended in Ringer

[1] This compound will be denoted throughout by the term "catechol X" and is measured colorimetrically as the difference between the total catechol (determined by the ammonium molybdate method of Rae [1930]) and the adrenaline [Schild, 1933], using adrenaline in both cases as the standard.

solution alone were similarly carried out, the final assay for these being equivalent to the amount of adrenaline diffusing from the slices under the conditions described.

At the end of the experiment the tissue slices were withdrawn and allowed to drain into the solution before being discarded. To each tube was added 1 ml. 2·5 % uranium acetate (slightly acid) to precipitate protein, followed by 1 ml. 2 % Na_2HPO_4 to remove excess of the reagent. The mixture was then filtered and the adrenaline in the filtrate estimated colorimetrically [Schild, 1933]. The results are summarized in Table 1, in which the total amount of adrenaline formed in each tube or set of similar tubes is recorded as the amount finally

Table 1. *The effect of surviving adrenal tissue on the unidentified catechol derivative present in adrenal extract*

Exp.	Tissue	Tube	Adrenaline (mg.) present in final solution	Mean	Initially present in the solution X added: Adrenaline mg.	Initially present in the solution X added: Catechol X mg.	Adrenaline formed mg.
1	80 mg. medulla	Controls	0·286 0·233	0·260	0	0	—
		A	0·432	0·432	0·142	0·150	0·032
		B	0·577 0·540	0·559	0·213	0·225	0·086
		C	0·613	0·613	0·284	0·300	0·069
		D	0·617	0·617	0·355	0·375	0·002
2	85 mg. medulla	Controls	0·228 0·278	0·253	0	0	—
		A	0·362 0·386	0·374	0·104	0·154	0·017
		B	0·457 0·469	0·463	0·156	0·231	0·054
		C	0·547 0·478	0·513	0·208	0·308	0·052
		D	0·607 0·650	0·629	0·260	0·385	0·116
3	80 mg. medulla	Controls	0·255 0·284 0·280 0·270 0·282	0·274	0	0	—
		A	0·345 0·324 0·369 0·378	0·354	0·045	0·03	0·035
4	80 mg. cortex	Control	—	0	0	0	—
		A	—	0·059	0·078	0·09	0
		B	—	0·105	0·130	0·15	0
		Blank*	—	0·047	0·065	0·075	0
5	80 mg. cortex	Control	0·037 0·025 0·027	0·030	0	—	—
		A	0·544 0·530	0·537	0·614	—	0
		B	0·635 0·609	0·622	0·818	—	0

* Showing the effect of O_2 alone on the adrenal extract in the absence of tissue.

present in the solution less (*a*) the control value and (*b*) that present initially in solution X, depending on the amount of the latter added in each case.

Inspection of these figures indicates that when the gland extract is incubated as described with medulla slices there is a definite production of "adrenaline" which cannot be observed in parallel experiments conducted with cortex. The validity of the estimations will be discussed later: for the present the chromogen responsible for the colour reaction may be regarded as adrenaline.

In Exp. 1, in which the concentration of catechol substrate (X) varied between 3·0 and 7·5 mg./100 g., the effect obtained appeared to pass through a maximum (tube B) at which 0·086 mg. adrenaline was formed from 0·225 mg. catechol X, corresponding approximately, weight for weight, to a 40 % conversion. In Exp. 2, over a similar range of concentration, the conversion increased progressively up to 30 %. At this stage however, on account of the variability in the composition of the initial extract (fresh samples requiring to be made from different batches of gland for each experiment), especially in regard to the relatively high concentration of other possibly toxic extractives, it seemed inadvisable to attempt anything more than a clear demonstration of a synthesis without relating it to other factors. From this point of view, the most serious variation occurs in the amounts of adrenaline diffusing from the slices (i.e. the control values). Even if the least favourable control value be accepted, however (e.g. 0·278 mg. instead of the mean value 0·253 mg. in Exp. 2), three out of four instances show a significant rise in the adrenaline content of the solutions. Accordingly in Exp. 3 the same value of substrate concentration was taken in each of four tubes and likewise five separate determinations of the control value were made. A conversion approaching 100 % was recorded, although in this case the low initial concentrations in the gland extract did not allow of the same accuracy being attained as before.

The reality of these figures is emphasized however by comparison with those of Exps. 4 and 5, in which cortex slices were used. The figures recorded, which are the mean values of triplicate determinations, indicate only a destruction of adrenaline despite the protective effect afforded by ascorbic acid which is present in cortex in greater concentration than in medulla, and thereby stress the positive role played by the medulla. Apart from the control experiments, "blank" determinations were made in Exp. 4 in which adrenal extract, diluted with an equal volume of Ringer solution, was submitted to the action of O_2 in the absence of tissue—but without any positive effect. Comparison may be made with the similar negative results obtained in experiments on autolysing adrenal tissue outlined in Table 3, which are referred to later.

2. *Experiments with known substrates*

In their attempts to effect the synthesis of adrenaline from known substrates, Schuler & Wiedemann [1935] showed that slices of guinea-pig adrenal respiring under physiological conditions had no appreciable effect on phenylalanine, tyrosine, hordenine (*p*-hydroxyphenyldimethylethylamine), or phenylhydroxyethylamine; phenylethylamine gave a faint inconclusive response and only with tyramine was there a pronounced conversion into adrenaline. Moreover, this change was effected only by medullary tissue and not by cortex.

In the experiments with bovine adrenal medulla tissue outlined in Table 2, the same compounds (with the substitution of *N*-methyltyrosine for phenylhydroxyethylamine) were investigated by means of the technique described in the previous section. They were either satisfactory commercial samples of the

Table 2. *The effect of surviving adrenal tissue (medulla) on various substrates*

Each tube contained 80 mg. medulla tissue

Exp.	Substrate	Present in 5 ml. solution (mg.) Substrate (initial)	Adrenaline (final)	Mean	Adrenaline formed mg.
6	Control	—	0·339 0·401 0·371	0·370	—
	Tyrosine	1·00 0·60 0·40	0·372 0·358 0·352	0·361	0
	Methyltyrosine	1·00 0·60 0·40	0·371 0·395 0·357	0·374	0
	Hordenine	1·20 0·72 0·48	0·376 0·398 0·371	0·382	0
	Phenylethylamine	1·20 0·72 0·48	0·490 0·517 0·509	0·505	0·135
	Phenylalanine	1·00 0·60 0·40	0·388 0·408 0·421	0·406	0·036
7	Control	—	0·264 0·238 0·248 0·257	0·252	—
	Phenylethylamine	1·20 0·96 0·72 0·48	0·351 0·297 0·290 0·237	0·294	0·042
	Phenylalanine	1·00 0·80 0·60 0·40	0·271 0·232 0·291 0·250	0·261	(0·01?)
8	Control	—	0·273 0·311 0·283 0·289	0·289	—
	Tyramine	1·00 1·00 0·80 0·80 0·60	0·361 0·345 0·347 0·305 0·252	0·322	0·033
9	Control	—	0·299 0·302 0·251 0·266	0·280	—
	Phenylethylamine	0·90 0·90 0·90 0·90	0·380 0·319 0·312 0·357	0·342	0·062
	Tyramine	1·00 1·00 1·00 1·00	0·325 0·298 0·277 0·289	0·297	(0·017?)

salts[1] (hydrochloride or sulphate) or synthetic specimens suitably purified, dissolved in HCl and brought to *p*H 6·5. They gave no response to the Vulpian reaction (development of a red colour, on oxidation with I_2, which is the basis of the adrenaline estimation employed) or to the catechol reaction with ammonium molybdate. The concentration of the substrate solutions (equimolar) was 20–24 mg./100 ml., this figure being chosen as the maximum because a possible 30 % conversion into adrenaline would have been roughly equivalent to the amount of adrenaline diffusing from the medulla slices in the control tubes and therefore easily detectable. In order to allow for the possible toxic effects of such concentrations, the composition of the test solutions in each set of tubes was varied between the limits (5 ml. substrate without added Ringer-glucose solution) and (2 ml. substrate + 3 ml. Ringer-glucose solution), the substrate solutions being adjusted to the composition of Ringer-glucose solution immediately before use. The experiments were run for $2\frac{1}{4}$–$2\frac{1}{2}$ hr., using a 95 % O_2–5 % CO_2 mixture for the respiration of the slices.

Similar tests were also made with dopa (dihydroxyphenylalanine) and methyldopa, but as these compounds give a positive Vulpian reaction the solutions were assayed biologically.

In some of these experiments the total amount of tissue employed necessitated using material from more than one gland and the length of time required for its preparation made it difficult to ensure that each batch of tubes contained reasonably uniform material in a state of satisfactory respiration. As far as possible, therefore, the slices were selected in such a way as to avoid the accumulation of possibly deteriorated tissue or of grossly different tissue in any one set of tubes.

As the results do not seem to bear any obvious relation to the variation in the concentration of substrate employed, only the mean value of the figures for each set of tubes need be considered. Bearing in mind the variation in the control values as indicative of the range of experimental error, it will be seen that in Exp. 6 tyrosine, methyltyrosine and hordenine showed no response, but that a positive effect was obtained with phenylethylamine and phenylalanine in the order named, the relative efficiency of conversion into the chromogen for these two compounds being in the ratio 4 : 1 approximately. A re-examination of these two substances (Exp. 7) again showed a positive but much smaller effect, that for phenylalanine (0·01 mg.) being no greater than for hordenine in Exp. 6, which was not considered significant. As the figure for phenylethylamine was only 0·042 mg., however, a value not greater than 0·01 mg. was to be expected for phenylalanine on the basis of the previous results. Presumably physiological conditions were not reproduced with such success as in the first trial. Exps. 8 and 9 confirmed these results in the case of phenylethylamine, and showed that tyramine, like phenylalanine, also gives a small but definite response.

It is clear that the most striking effect is shown by phenylethylamine, the maximum conversion being represented by Exp. 6 in which 3 tubes containing a total of 2·4 mg. substrate yielded altogether $3 \times 0{\cdot}135$ mg. adrenaline, equivalent to a 17 % return. This is probably a minimum figure in so far as the optimum concentration of substrate would appear to be much less than the highest in the range employed. Tyramine and phenylalanine show a smaller but nevertheless real effect, while tyrosine, methyltyrosine and hordenine do

[1] The writer is greatly indebted to Prof. H. S. Raper who supplied samples of tyrosine, hordenine, dopa, methyldopa and methyltyrosine, and to Prof. H. J. Channon for B.D.H. samples of β-phenylalanine, β-phenylethylamine and tyramine.

not respond at all. As in the experiments on adrenal extract detailed in the first section, blank tests were carried out on phenylethylamine and phenylalanine in which the tissue slices were omitted while O_2 was bubbled through the solutions. In some cases known amounts of adrenaline and ascorbic acid were added to simulate the conditions when tissue was present. In none of these trials, however, was any positive effect obtained.

The experiments with dopa and methyldopa were completed with the assistance of Miss M. Fleure, to whom the writer is greatly indebted. Pressor assays of the final solutions were carried out on a pithed cat, but no appreciable variation from the control values could be detected. This confirms and extends the observation of Schuler & Wiedemann on dopa.

3. *The effects of autolysis of adrenal tissue*

Reference has already been made to the negative results obtained with autolysing tissue. Hallé [1906] brought forward evidence from gravimetric assay to illustrate the production of adrenaline from tyrosine when the latter was incubated under aseptic conditions with minced adrenal tissue. Ewins & Laidlaw [1910], employing a more accurate physiological assay, were unable to confirm these results either with tyrosine or tyramine. More recently, Larrain *et al.* [1934] claimed that a collodion dialysate of autolysing adrenal tissue contains a surprisingly large quantity of an adrenaline-like substance, with a higher physiological than chemical assay. A further experiment along these lines was therefore devised in which the effect of incubating both medullary and cortical tissue, with and without the addition of tyramine, was examined colorimetrically.

Table 3. *Effect of autolysis on the adrenaline and total catechol content of adrenal tissue in the presence of tyramine*

Period of incubation hr.	Medulla				Cortex			
	Control		Experimental		Control		Experimental	
	Total catechol	Adrenaline	Total catechol	Adrenaline	Total catechol	Adrenaline	Total catechol	Adrenaline
0	13·6	10·0	—	—	1·2	0·6	—	—
19	14·2	9·0	13·8	10·2	1·1	0·5	0·9	0·4
25	14·2	10·3	13·4	10·0	—	—	—	—
52	—	8·0	—	8·2	—	—	—	—
66	—	—	—	—	1·0	0·3	0·9	0·3
90	—	—	—	—	1·0	0·3	0·9	0·3

For this purpose quantities of 5·5 g. medulla and 12 g. cortex tissue from fresh bullock adrenals were weighed into bottles from a homogeneous mash of each tissue and diluted to 50 ml. with water slightly acidified with acetic acid. Each set was divided into two groups, to one of which tyramine hydrochloride was added (50 mg. to medulla and 20 mg. to cortex). After incubation at 37° for varying periods under toluene, the experimental and control samples (the latter without added tyramine) were twice extracted with trichloroacetic acid and assayed colorimetrically for adrenaline and total catechol. By comparing the figures obtained with those for a direct extract of a sample of the original tissue pulp, it was possible to determine whether any chromogenic substance had been generated merely by the process of autolysis or whether such an effect could arise from the action of autolysis on a compound closely related to adrenaline, such as tyramine. The evidence summarized in Table 3 indicates that no

substantial effect of this kind is demonstrable either with cortex or medullary tissue. The catechol test alone suffices to show that under these conditions the phenolic nucleus of the tyramine molecule does not undergo the further oxidation which is, as will be seen, a necessary preliminary to the formation of adrenaline or any "adrenaline-like" substance which responds to the Vulpian reaction.

Discussion

The main point arising out of the present work, which was undertaken primarily to test the general applicability of Schuler & Wiedemann's finding that tyramine is the precursor of adrenaline, is that in the adrenal of the bullock, as far as can be determined by colorimetric analyses alone, this function appears to be taken over by the analogous compound β-phenylethylamine. Both sets of results are conveniently tabulated below for comparison.

	Response to adrenal tissue	
Substrate	Guinea-pig (Schuler & Wiedemann)	Bullock (Present paper)
Tyrosine	0	0
Phenylalanine	0	+
Hordenine	0	0
Phenylhydroxyethylamine	0	Not tested
Phenylethylamine	+	+ +
Tyramine	+ + +	+
Dopa	0	0
Methyldopa	Not tested	0
Methyltyrosine	,,	0
[Adrenal extract	,,	+ +]
	Effect confined to surviving medullary tissue; not shown by cortical tissue or by powdered or pulped medulla (autolysis). Colorimetric determination (Folin) "confirmed" by pressor assay	Effect shown by surviving medullary tissue; autolysing medulla or cortical tissue alone or with added tyramine shows no effect. Effect on adrenal extract (catechol X) obtained only with medullary tissue, not with cortex. Colorimetric determination [Schild]

+, + +, etc. indicate approximately the extent of the responses in each group; 0 indicates no significant effect.

These results bear a superficial resemblance to one another in so far as they show that in each case the substrate of highest activity is an unsubstituted amine (tyramine or phenylethylamine). This is in marked contrast to the *in vitro* experiments of Heard & Raper [1933], in which it was shown that when compounds such as dopa and methyldopa are submitted to the action of tyrosinase, retention of the terminal COOH group is necessary for β-oxidation to proceed in the side chain, this being an important stage in the development of pressor activity. The supposition that the conversion of the parent substance tyrosine or phenylalanine into adrenaline proceeds only via carboxyl derivatives (the COOH group inhibits pressor activity) was also a feature of Guggenheim's [1924] theory, since this would explain the apparent absence from the adrenal gland of any pressor substance other than adrenaline.

Consideration of the data presented by Schuler & Wiedemann shows that besides tyramine several other substrates exhibit some slight activity, notably in the case of phenylethylamine which in different experiments led to increases up to 100% in the adrenaline content of the test solutions over the control

values, as compared with increases of 45–400 % recorded for tyramine (measured colorimetrically). Although only the latter was checked by physiological assay, the effect with phenylethylamine is perhaps not insignificant, bearing in mind its analogous derivation from phenylalanine which is the only protein constituent other than tyrosine which suggests itself as the parent substance of adrenaline. In our experiments the activities were found to lie in the reverse order although at a lower[1] level; phenylethylamine showed the greatest activity while tyramine and phenylalanine were each about one-quarter as active. Granted a certain parallelism between the two sets of results, the question naturally arises as to how far such differences are attributable to deviations in technique. While the tests described in the present communication have been more limited in scope and the nature and *p*H of the medium, etc., have not been varied, there is no reason to suppose that such alterations towards less physiological conditions would have resulted in substantially different *relative* results. Nor does a study of the colorimetric analyses employed, which at first seems a more likely explanation, lead us any further. The Folin reaction for adrenaline, which is primarily a test of reducing power, is open to many grave objections [Devine, 1936] and is particularly liable to error in experiments dealing with the adrenal gland on account of the occurrence in this tissue of a notable amount of ascorbic acid, which even in its partially oxidized form is still capable of reacting with the reagent. If such sources of error be accounted for by an adequate control, the reaction becomes essentially a test for the reducing power of the catechol nucleus and hence of nuclear oxidation as, for example, in the conversion of tyrosine into dopa. The Vulpian reaction (and similar tests involving the production of a red pigment on oxidation of adrenaline), while being more specific, is likewise of limited value, since a positive test is readily given by any 3:4-dihydroxy derivative of tyrosine, i.e. with a primary or secondary amino-group in the β-position in the side chain, except when a keto-group occupies the α-position as in adrenalone [Ewins, 1910]. It seems probable that closely allied catechol compounds give almost identical responses in the oxidation test [Maiweg, 1922; Heard, 1932] so that results obtained on this basis with different substrates reflect closely the actual extent of the molecular changes involved in each case; it seems less certain from the observations of Barker *et al.* [1932] that this can be claimed also for the Folin test. Moreover, if compounds such as ascorbic acid, to which both in the oxidized and reduced forms Folin's reagent is sensitive, play an active part in the synthesis, then the value of a control test in which this point would not operate might be seriously diminished, thus making the possibility of correlating the chemical and physiological assays, which must be the final criterion, even less likely. In mentioning the inadequacy of their results when viewed in this light, Schuler & Wiedemann do not claim the end-product of their *in vitro* experiments with tyramine to be more than "adrenalinartige Substanz". It is clear that for further clarification of this point some proved form of the Vulpian reaction must be used [cf. Euler, 1933; Schild, 1933] in conjunction with physiological assay. The results presented in this paper form a basis for such an investigation.

The observations recorded in Table 1 on the response of adrenal extracts (catechol X) to the action of respiring adrenal tissue lend experimental support for the first time to the probability that this unidentified compound represents

[1] The advantage of preparing sufficient material to make an adequate number of tests on several substrates simultaneously is to some extent counterbalanced by the possible loss of activity due to deterioration of the tissue, especially when the latter is not derived from a readily available laboratory animal.

a definite stage in the *in vivo* formation of adrenaline. In a short note, which does not appear to have been enlarged upon later, Kendall [1932] mentions the existence in the adrenal gland of a lactic acid derivative of adrenaline (epinephrine)—"lactyl epinephrine" which is resistant to hydrolysis and which reduces Folin's reagent and gives the catechol test with $FeCl_3$. In the absence of further detail it need only be said that while such a derivative of preformed adrenaline might explain the existence of catechol X, it seems unreasonable to suppose that it is so unstable under the mild conditions of the incubation as to provide an explanation of the increase in the colorimetric assay. Another possibility is that catechol X is closely related to adrenaline but possesses in the side chain an inhibitory group which does not permit of a positive Vulpian reaction. Since the —CO group possesses this property and it has already been established by Heard & Raper [1933] that adrenalone[1] $(OH)_2C_6H_3.CO.CH_2.NHCH_3$ is produced by the *in vitro* action of tyrosinase on *N*-methyldopa, it would be of great interest to determine physiologically how adrenalone behaves in the presence of surviving adrenal tissue. An attempt was made to test this point colorimetrically as for the other substrates but the immediate oxidation of the excess adrenalone by I_2 completely masked any red coloration which may have been present. Heard & Raper were unable to obtain any evidence that adrenalone is reduced to adrenaline when it is perfused through the surviving adrenal gland, although the conditions are favourable towards reduction; perfusion of dopa and methyldopa resulted in their being stabilized against subsequent oxidation. As yet, therefore, the evidence from both perfusion and tissue slice experiments is against accepting either of these compounds as intermediates, although the difficulty of ensuring truly physiological conditions in such tests must be realized.

Summary

1. Experiments have been carried out in which solutions of various substrates related to tyrosine were submitted to the action of surviving tissue slices of the bullock adrenal medulla under physiological conditions and then assayed colorimetrically for adrenaline.

2. Of these compounds phenylethylamine was the most active, being converted in amounts up to nearly 40% into an adrenaline-like substance (i.e. responding to the Vulpian reaction) while tyramine and phenylalanine were about one-quarter as active. The results with tyrosine, methyltyrosine and hordenine were negative, as also with dopa and methyldopa (determined physiologically).

3. A marked response was also given by the unidentified catechol compound present in adrenal extracts. This effect is limited to medulla tissue and is not obtained with cortex.

4. Autolysis of cortex or medulla tissue over a period of many hours does not give rise to adrenaline. The result is not affected by the presence of tyramine.

5. These results are discussed, particularly in relation to the claim of Schuler & Wiedemann [1935] that tyramine is the precursor of adrenaline in the guinea-pig adrenal.

The author is very grateful to Prof. H. S. Raper for his continued interest in the work and for the supply of specimens, and also to Miss M. Fleure for

[1] A compound such as adrenalone which possesses only 1/100th the pressor activity of *l*-adrenaline would easily escape detection by physiological means if present in the adrenal gland in concentration comparable with that of catechol X.

assistance in making pressor assays. The investigation was completed through the kindness of Prof. H. J. Channon, who provided laboratory facilities and material, and to whom the writer is especially indebted.

REFERENCES

Barker, Eastland & Evers (1932). *Biochem. J.* **26**, 2129.
Devine (1936). *Biochem. J.* **30**, 1768.
—— (1937). *Biochem. J.* **31**, 545.
Dulière & Raper (1930). *Biochem. J.* **24**, 239.
Euler (1933). *Biochem. Z.* **260**, 18.
Ewins (1910). *J. Physiol.* **40**, 317.
—— & Laidlaw (1910). *J. Physiol.* **40**, 275.
Guggenheim (1924). *Die biogenen Amine.* 2nd Ed. Berlin: Springer.
Quoted by Heard [1932].
Hallé (1906). *Beitr. chem. Physiol. Path.* **8**, 276.
Heard (1932). Thesis. Manchester.
—— & Raper (1933). *Biochem. J.* **27**, 36.
Kendall (1932). *J. biol. Chem.* **97**, 4 *P.*
Larrain, Roberts & Kunde (1934). *Proc. Soc. exp. Biol., N.Y.*, **31**, 534.
Maiweg (1922). *Biochem. Z.* **134**, 292.
Rae (1930). *Pharm. J.* **125**, 451.
Raper (1927). *Biochem. J.* **21**, 89.
Schild (1933). *J. Physiol.* **79**, 455.
Schuler, Bernhardt & Reindel (1936). *Hoppe-Seyl. Z.* **243**, 90.
—— & Wiedemann (1935). *Hoppe-Seyl. Z.* **233**, 235.

LINDA HALL LIBRARY
KANSAS CITY, MO.

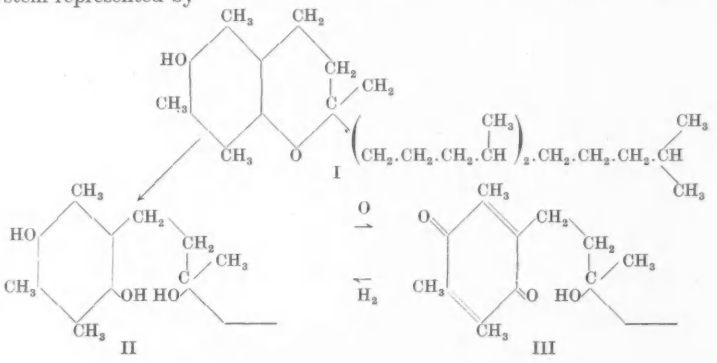
5. ON THE BIOLOGICAL SIGNIFICANCE OF THE TOCOPHEROLS (VITAMIN E)

By MARGARET D. WRIGHT and JACK CECIL DRUMMOND

From the Research Laboratories, Vitamins Ltd., and Department of Biochemistry, University College, London

(*Received 9 November 1939*)

The readiness with which the tocopherol molecules are oxidized to hydroxyquinones [Moss *et al.* 1938; John, 1938] raises the interesting speculation whether there may exist in animal tissues as part of the oxido-reduction mechanisms a system represented by



Such a system would place α- and β-tocopherols in the category of the provitamins, the open-ring derivatives being the biologically important substances [Drummond, 1939]. In this connexion we had been impressed by the rapid disappearance of tocopherols from animal tissues [Cuthbertson *et al.* 1939].

Against the view that there may be a reversible quinol-quinone system originating in the tissues from tocopherols are the failures to substitute vitamin E in the diet by the quinone [Karrer *et al.* 1938; John *et al.* 1939] and our inability to detect the quinone by spectroscopic methods in extracts of the tissues of animals which had been given large doses of vitamin E [Cuthbertson *et al.* 1939].

It was necessary, however, to be certain that the negative results obtained in these experiments were not due to failure to absorb the quinone when given orally.

Accordingly, comparative tests have been made in which α-tocopherol and its hydroxyquinone (III) were administered in equal amounts by subcutaneous injection into pregnant rats on a vitamin E-deficient diet.

The quinone was prepared from α-tocopherol by oxidation for a few minutes at 65° with 5% silver nitrate dissolved in 90% methyl alcohol. This method is more satisfactory than that described by John [1938].

No. of rats	Dose	Effect
3	5 mg. α-tocopherol acetate	2 resorptions 1 litter
6	10 mg. α-tocopherol acetate	3 resorptions 3 litters
3	5 mg. quinone	All resorbed
5	10 mg. quinone	All resorbed

These results seem to show that the quinone is not biologically active when administered in this manner in amounts which should be effective were such a reversible system part of the oxidation mechanisms of the tissues.

Part of the expenses of this research were defrayed by a grant from the Medical Research Council which one of us (J. C. D.) wishes gratefully to acknowledge.

REFERENCES

Cuthbertson, Ridgeway & Drummond (1940). *Biochem. J.* **34**, 34.
Drummond (1939). Vitamin E Monograph. Society of Chemical Industry.
John (1938). *Hoppe-Seyl. Z.* **252**, 222.
—— Dietzel & Emte (1939). *Hoppe-Seyl. Z.* **257**, 180.
Karrer, Salomon & Fritzsche (1938). *Helv. chim. Acta*, **21**, 309.
Moss, Cuthbertson, Danielli & Drummond (1938). *Chem. Ind. Rev.* **58**, 275.

6. THE FATE OF TOCOPHEROLS IN THE ANIMAL BODY

By WILLIAM FRANCIS JACK CUTHBERTSON, RADFORD ROY RIDGEWAY and JACK CECIL DRUMMOND

From the Department of Biochemistry, University College, London

(*Received 9 November 1939*)

Very little is recorded in the literature regarding the fate of the tocopherols in the animal body. The original studies of Evans & Burr [1927] gave strong indications that vitamin E is not stored in tissues to any significant extent and also that such reserves as there may be are gradually reduced as a result of normal metabolic processes when the diet is deficient in that respect. They emphasized that the rate of utilization was not increased by gestation, as might perhaps have been expected.

We have attempted to study the fate of the tocopherols of wheat germ oil by a series of metabolism experiments followed by the examination of the tissues of the animals.

Experiment 1

Two groups, each of 4 adult bucks and 4 does, were compared. One had been fed on a vitamin E-deficient diet for 4 months after weaning. The other had had an adequate diet for the same period. The compositions of the two diets were:

	Vitamin E-deficient	Adequate
Extracted casein (Glaxo)	18	18
Rice starch	45	45
Lard	12	12
Yeast extract (Yeastrel)	8	8
Cane sugar	10	10
Salt mixture	5	5
Cod-liver oil	2	—
Cottonseed oil + 1 % halibut-liver oil	—	2

Each group of rats was kept in a metabolism cage and records were kept of food consumption, body weight etc. After a preliminary period of 3 days, urine and faeces were collected daily over a period of a week; great care was taken to avoid any contamination of either by food particles.

The faeces were ground with anhydrous Na_2SO_4 and the resulting powder was fully extracted with purified ether. The urines were brought to approximately *p*H 1 and extracted with ether in a continuous-extraction apparatus for 6 hr. The ether was removed from the extracts of urine and faeces and the residues were extracted with light petroleum until no more material went into solution.

Spectroscopic examination of the petroleum-soluble fractions, representing periods of a week for each group, both before and after chromatographic adsorption on aluminium oxide columns failed to reveal any selective absorption which might be taken as indicating the presence of tocopherols. The fractions from the urine extracts showed strong absorption in the region 270–280 mμ which was traced to the presence of phenolic substances easily removed by washing with

dilute alkali. Their removal did not unmask any recognizable absorption likely to be due to tocopherols.

Each animal was then given a daily dose of a wheat germ oil concentrate containing approximately 45 % of the natural mixture of α- and β-tocopherols. The concentrate was diluted with 3 parts of olive oil to facilitate dosing and each rat received an amount equivalent to 3·5 mg. of tocopherols daily for a week, the measured dose being given directly into the mouth by means of an Agla syringe.

Spectroscopic examination of extracts of the urines failed to show any trace of tocopherols, even after the extracts had been fractionated by chromatographic adsorption. The fats from the faeces gave clear indications of vitamin E which had presumably escaped absorption from the intestine. Estimates placed this amount at from 3 to 15 % of the total amount administered over the whole week, an interesting point being that the males consistently excreted a larger proportion (10–15 %) than the females (3–6 %).

At the conclusion of the experiment the animals were killed and tissues were removed from them and dehydrated by grinding with anhydrous Na_2SO_4. Ether extracts, after removal of the solvent and spectroscopic examination in *cyclo*hexane solution, did not show selective absorption related to vitamin E in the case of the heart, kidney, adrenals, testes, ovaries, liver or pituitary. Extracts of leg muscles and of body fat showed definite evidence of the presence of tocopherols.

It may be useful to record some of our observations on the spectroscopic examination of extracts of animal tissues for the tocopherols. Small amounts of these substances may easily be missed when they are insufficient (i.e. less than about 0·1 %) to produce an inflexion in the curve representing the ultra-violet absorption of the fat itself. In all such doubtful cases the unsaponifiable fraction, prepared with care to minimize losses by oxidation, should be examined.

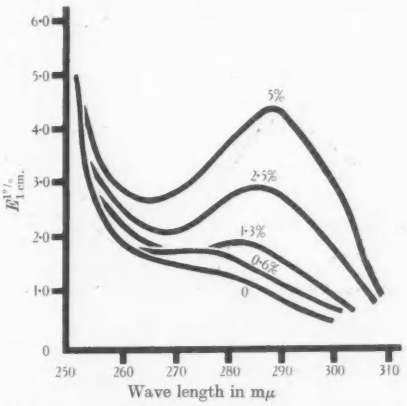


Fig. 1. Influence of different amounts of α-tocopherol on the shape of the absorption curves of rat kidney fat.

Amounts of tocopherols sufficient to give rise to distinct inflexions in the absorption curves of the crude extracts from tissues do not always reveal themselves by maxima at 290–294 mμ. Low concentrations may influence the curve

in the vicinity of 270–280 mμ. With increasing concentration the position of the maximum tends to shift towards the region characteristic of the tocopherols. This shift is illustrated by the curves in Fig. 1. In our experience the direct spectroscopic estimation of tocopherols in crude tissue extracts is unreliable if the proportion is much below 1%, but examination of the unsaponifiable fraction will enable quantities down to about 0·1% to be determined satisfactorily. Actually, however, we have found it advisable, whenever relatively small amounts are likely to be present, to oxidize the unsaponifiable fraction

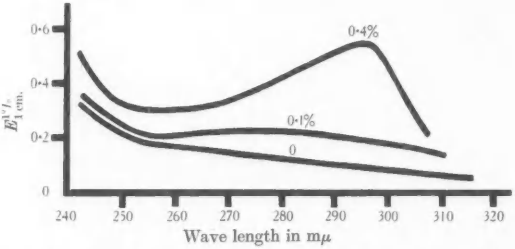


Fig. 2. Influence of 0·1 and 0·4% of α-tocopherol on absorption curve of body-fat from rats on a vitamin E-deficient diet.

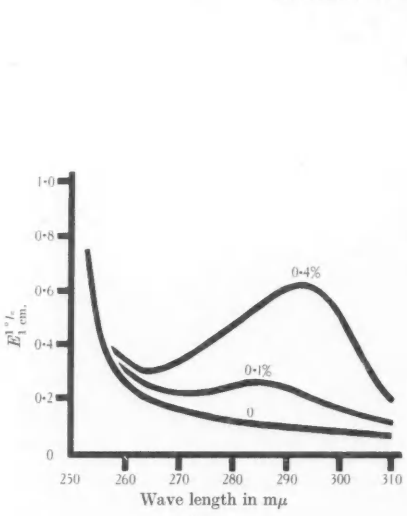


Fig. 3.

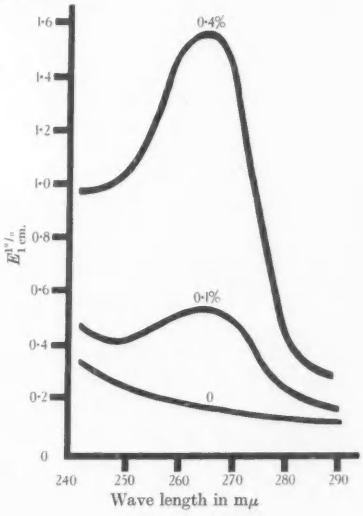


Fig. 4.

Fig. 3. Absorption curves of the unsaponifiable fraction of rat body-fats containing respectively 0, 0·1 and 0·4% of α-tocopherol.

Fig. 4. Absorption curves of oxidized unsaponifiable fractions of rat body-fat containing respectively 0, 0·1 and 0·4% α-tocopherol.

with silver nitrate so that the tocopherols are quantitatively converted into the quinones [Moss *et al.* 1938] which show well-marked maxima in the vicinity of 265 mμ with an intensity about 4 times as great as that of the parent substances.

This modification of the spectroscopic method enables one to detect as little as 0·01 % of a tocopherol in an unsaponifiable fraction and to estimate with reasonable accuracy proportions above 0·03 %. For oxidizing the unsaponifiable fractions we employed a 5 % solution of silver nitrate in 90 % methyl alcohol acting at 65° for only 5 min. This method appears to have advantages over that employed by John [1938] in giving a cleaner material and a better yield. The spectroscopic examination of samples of rat body-fat with added tocopherol and of the corresponding unsaponifiable fractions before and after oxidation are shown in Figs. 2–4.

The detection of traces of tocopherols in extracts of the leg-muscles and of significant amounts in the body-fat of the rats employed in Exp. 1 recall the observations of Evans & Burr [1927].

Experiment 2

In this metabolism experiment a group of 10 rats which had previously been fed for 12 months on a diet unusually rich in natural tocopherols was examined. They received the same diet during the period of 3 weeks that they were under study. The diet was composed of:

Casein	18
Rice starch	48
"Fertilol" (wheat germ oil of tested potency)	12
Olive oil	8
Dried yeast	10
Salt mixture	4
Cod-liver oil	Bi-weekly dose to provide vitamin A

We again found evidence of incomplete absorption, for the faecal fats accounted for approximately 25 % of the tocopherols contained in the basal diet.

The examination of the urines from this group of rats gave very interesting results. To simplify spectroscopic examination and to facilitate separation from phenolic substances the fats were saponified carefully under conditions giving protection against oxidation.

Material was present which absorbed selectively in the vicinity of 290 mμ and was recognized as tocopherol. Estimations indicated that the urine passed by 10 adult rats fed on this vitamin E-rich diet for 19 days contained altogether about 6–8 mg. of tocopherols.

Colorimetric estimations carried out on the fractions by use of the ferric chloride-αα-dipyridyl method of Emmerie & Engel [1938; 1939, 1] gave values of the same order. It seems established, therefore, that tocopherols pass into the urines of rats in small quantities when their diet is very rich in that respect.

Spectroscopic examination was made of extracts of tissues removed from these rats. It revealed storage of vitamin E in the body-fat (approximately 0·04 %) and the presence of traces in the blood. Other tissues gave negative results, including the liver.

We were at first in doubt whether it would be possible easily to detect vitamin E by spectroscopic means in extracts, such as those from livers, which might show considerable absorption at or near 328 mμ as a consequence of the presence of vitamin A. This was put to direct test by examining rat liver fat to which known quantities of vitamin A and α-tocopherol acetate had been added. It was found that quantities of the latter of the order of 0·02 % were detectable.

Extracts of the livers of the rats of Exp. 2 showed no sign of absorption at or near 263 mμ after oxidation of the unsaponifiable fraction with silver nitrate under the conditions we have described. We support, therefore, the view of Moore *et al.* [1939] that storage of vitamin E in the liver does not occur when rats are fed on a diet rich in tocopherols. This method enabled us to detect traces of tocopherols in the blood of these rats, but the amounts were too small to be estimated with any reliability by the spectroscope. They were probably of the same order as the concentrations (0·03–0·09 mg./100 ml.) found by Emmerie & Engel [1939, 2] in the serum of rats after oral administration of doses of 1–5 mg. of tocopherol.

Our detection of what appeared to be vitamin E in the urine of rats which had been living for a long time on a diet unusually rich in that substance had some bearing on a curious observation made by Jones [1936; 1937; 1938] in certain studies on the excretion of oestrogens by pregnant women. Following up the discovery that in certain cases there were marked discrepancies between the colorimetric estimation of these compounds and the values given by spectroscopic assays, he reached the conclusion that in a normal pregnancy there is a substance in the urine which shows absorption in the same region of the ultraviolet as oestriol and oestrone and which may be responsible for the higher values which the spectroscopic method sometimes gives. He attempted to isolate this substance and at length obtained from the toluene residues yielded by Marrian's well-known process for the separation of urinary oestrogens a few mg. of a crystalline material, M.P. 225–230°, possessing some oestrogenic activity and showing marked selective absorption at 294 mμ. He suspected that it might be vitamin E or a closely related compound, not only because of its spectroscopic properties but because it appeared to be absent from urines in cases in which intra-uterine death had occurred.

We have been unable to confirm his more important claims. Quantities of the order of 15 l. of urine from women in the latter part of pregnancy were evaporated down to 1 l. and hydrolysed with HCl at *p*H 1 by heating in the autoclave for 2 hr. at 15 lb. pressure. The residue was extracted with ether and the extract was washed, first with several quantities of 10% Na_2CO_3 and secondly with several changes of $N/10$ NaOH. The washed ether layer was freed from solvent, taken up in toluene and extracted several times with N NaOH. The toluene-soluble residue was a bright red, viscous oil which on recrystallization from ethanol yielded a few mg. of a crystalline substance with a low melting point, but which was spectroscopically transparent. The residual solution on evaporating down and cooling yielded a further crop of crystalline material. This proved, after several recrystallizations from alcohol, to be a compound similar to that isolated by Jones. It melted at 228–229° and, although not purified fully, was almost certainly impure pregnandiol. It was spectroscopically transparent.

Examination of the mother liquors showed distinct signs of selective absorption in the vicinity of 280 mμ. The thick, red, oily material was, therefore, dissolved in benzene and subjected to chromatographic adsorption on aluminium oxide. Several fractions showed on spectroscopic examination pronounced bands between 280 and 290 mμ which seemed to indicate the presence of tocopherols or closely related substances. Estimations were then made on all the fractions by the colorimetric method of Emmerie & Engel [1938; 1939, 1]. The reducing powers of the extracts corresponded to amounts of tocopherols approximately half those indicated by the spectroscopic data. This was probably due to interference caused by other substances absorbing in the vicinity of 280 mμ.

The whole of the fractions representing 15 l. of urine from late pregnancy cases contained about 10 mg. of material estimated as tocopherols by the reduction method.

It appears reasonably certain, therefore, that the material isolated by Jones was impure pregnandiol, contaminated with traces of oestrogens and vitamin E. McArthur & Watson [1939] have recently reported their failure to detect vitamin E in fractions separated from the ether extract of 40 gallons of non-pregnant cows' urine.

It seems clear from these results, taken as a whole, that the tocopherol molecule is readily broken down in the body of the rat and that storage occurs in the adipose tissues only when the diet provides an unusually high intake of vitamin E. The results of the experiments do not give any indication of the function of the vitamin in the tissues.

Mr G. Napier gave valuable help in making the examination of liver fats to which known quantities of vitamins A and E had been added.

Part of the expenses of this research were defrayed by a grant from the Medical Research Council which one of us (J. C. D.) wishes gratefully to acknowledge.

We also wish to thank Roche Products Ltd. for a generous gift of α-tocopherol acetate to assist in this work.

REFERENCES

Emmerie & Engel (1938). *Rec. Trav. chim., Pays-Bas*, **57**, 1351.
—— —— (1939, 1). *Rec. Trav. chim., Pays-Bas*, **58**, 283.
—— —— (1939, 2). *Rec. Trav. chim., Pays-Bas*, **58**, 895.
Evans & Burr (1927). *Mem. Univ. Calif.* no. 8.
John (1938). *Hoppe-Seyl. Z.* **252**, 222.
Jones (1936). Dissertation, Liverpool.
—— (1937). *Lpool med.-chir. J.* **45**, 112.
—— (1938). *J. Obstet. Gynaek.* **45**, 281.
McArthur & Watson (1939). *Canad. Chem. Proc. Ind.* **23**, 350.
Moore, Martin & Rajagopal (1939). Vitamin E Monograph. Society of Chemical Industry.
Moss, Cuthbertson, Danielli & Drummond (1938). *Chem. & Ind.* **133**, 275.

7. THE INFLUENCE OF PROLONGED STARVATION ON THE COMPOSITION OF PIG DEPOT FATS

By THOMAS PERCY HILDITCH and WILLIAM HOPPER PEDELTY

From the Department of Industrial Chemistry, University of Liverpool

(*Received 9 November 1939*)

Until recently, little or no information has been published with reference to the rates at which the individual component acids of a depot fat disappear during starvation. Work in this field of a strictly quantitative nature is lacking except for recent studies by Longenecker [1939, 1, 2, 3] detailed below. Dowell [1920] raised rabbits on a peanut diet for 40 days, after which some were killed and others were starved for periods from 3 to 7 days. His results showed a fall in I.V. for the depot fats and also a preferential selection of the back fat over that surrounding the kidney. A similar diminution in I.V. was noted by Stefko [1928] in the subcutaneous and deeper fat tissue of tubercular and starved human subjects. Mann [1936] also showed that fat changes in hibernating bats were characterized by a rise in M.P. with corresponding fall in mean unsaturation. Lovern [1934; 1938] found that in the fasting herring the more unsaturated components of the fat appeared to be preferentially utilized; but in fasting salmon the average unsaturation was not greatly altered, except for some decrease in hexadecenoic acid. Frank [1926] concluded that in starved rats desaturation of saturated fatty acids accompanies β-oxidation of fatty acids.

The only other studies of the effect of inanition on depot fat composition are those of Longenecker on the rat. Longenecker [1939, 1] found that the composition of the total body fat of a normal rat after 28% loss in weight during starvation showed little significant alteration; a similar strain of animal after rearing on a high carbohydrate diet with subsequent starvation to 22% weight loss gave a body fat, which, when compared with the control, showed a decrease in total C_{16} acids (palmitic and hexadecenoic) with a corresponding rise in the amount of oleic and linoleic acids. In a second group of experiments [1939, 2], rats were fed on a high corn oil diet which produced a "soft" fat. Two groups were then fasted to 14 and 24% weight loss and the results showed that utilization (during inanition) of rat depot fat containing mainly oleic and linoleic acids did not produce marked changes in the relative proportions of these acids, little selection being evident from the available fatty acid mixture. A gradual increase in oleic acid content parallel with reduction in linoleic acid content occurred during fasting, but this might be regarded as within the limit of physiological variation rather than as an indication of a preferential utilization of the more highly unsaturated acids. In a third paper, Longenecker [1939, 3], describes similar fasting of rats fed for 14 days on a diet rich in coconut oil. After fasting to 15% loss in weight, lauric acid disappeared more rapidly than oleic acid; on further fasting to 30% loss in weight the same effect was observed, although the ratio of oleic : lauric acid utilized was higher at this stage. Longenecker points out that, whilst his data may indicate preferential utilization of acids of

lower mol. wt., they may equally be attributed to the random removal of triglyceride molecules from the depots, since most of these molecules contain two lauric (or other lower saturated acyl) groups, the third being oleic, myristic or palmitic.

The present investigation was undertaken to ascertain whether, during extreme starvation of pigs, any preferential utilization of individual saturated or unsaturated fatty acids occurs in the depot fats. Of all domestic animals the pig is the most suitable for studies of this nature, on account of its relatively large proportion of reserve fat. Attention was confined to the outer and inner back fats and the perinephric fats, which were clearly likely to undergo most marked alteration, since during starvation an animal uses its depot fats in preference to the more vital organ fats, the latter only being appreciably utilized in the final stages preceding death.

Experimental

As in our recent study of the deposition of component glycerides in back and perinephric fats of the pig [Hilditch *et al.* 1939], the present examination was made possible by access to the depot fats of pigs from an experiment conducted by Dr J. Hammond at the Animal Nutrition Institute of the School of Agriculture, Cambridge, on the changes occurring during fasting. This experiment consisted in rearing 5 pigs of homogeneous stock and sex (hogs) from weaning as rapidly as possible to approximately 330 lb. live weight on an unrestricted diet consisting of the following meal mixture: white fish meal 15%, flaked maize 40%, barley 20% and pollards 25%, with the addition of 1% of cod liver oil. One animal (no. 175) served as control and was killed as soon as it reached 330 lb. live weight; the other four were fasted (except for the provision of water containing essential mineral salts) and given exercise daily. Of these animals, only the fats of the 3rd and 5th animals to be killed have been studied in detail in the course of the present work. The pigs in question were no. 167, starved for 51 days, and the final animal, no. 173, which had fasted for 135 days and was quite active and apparently healthy at the time of death. Both animals lost weight much more slowly than had been expected and it is considered that pig

Table 1

Pig	Days fasted	Killing weight lb.	Loss in weight lb.	% loss in weight	Total weight of left loin and thorax g.	Weight of both perinephric fats g.
175	0	327	0	0	6671·5	3806·0
167	51	263	65	19·8	4149·0	2878·0
173	135	188	143	43·2	1754·3	1171·0

		Fresh tissue				Extracted fat		
Pig	Depot	Weight g.	Fat %	Tissue %	Water %	Weight g.	I.V.	% of pig 175
175	Outer	2928·0	87·1	2·2	10·7	2549	63·9	100
	Inner	3743·5	92·7	1·2	6·1	3472	58·9	100
	Perinephric	3806·0	93·5	1·2	5·3	3557	56·4	100
167	Outer	1729·0	87·1	2·9	10·0	1513	65·0	59·4
	Inner	2420·0	91·2	1·5	7·3	2208	59·9	63·6
	Perinephric	2878·0	93·0	1·3	5·7	2676·5	56·6	75·3
173	Outer	854·3	82·7	4·3	13·0	706·5	60·0	27·7
	Inner	900·0	86·3	2·6	11·1	776·7	54·7	22·4
	Perinephric	1171·0	87·4	2·8	9·8	1023·4	54·8	28·8

no. 173 would have lived considerably longer had time permitted. By the kind co-operation of Dr Hammond, we were supplied with the foregoing data and with the weights of fatty tissues, etc., at the time of slaughter as set out in Table 1. The tissues, from which the depot fats examined in this work were prepared, were handed to Drs E. H. Callow and C. H. Lea of the Low Temperature Research Station, Cambridge, who very kindly undertook the extraction (and storage at $-20°$ until required) of the fats dealt with in the analyses now recorded.

The component acids of the 9 depot fats from the 3 pigs (nos. 175, 167 and 173) were determined as already described [Hilditch *et al.* 1939]. After the preliminary division into "solid" and "liquid" acids by Pb salt separation from alcohol, the methyl esters of the "solid" acids were distilled from a Willstätter flask, and those of the "liquid" acids through an electrically-heated and packed column of the type described by Longenecker [1937]. The final weight and molar percentages determined for the acids from each depot fat are given in Table 2.

Table 2. *Component acids of control and starved pigs*

(*a*) Weight % (excluding unsaponifiable matter)

	Pig 175			Pig 167			Pig 173		
Acid	Outer	Inner	Peri-nephric	Outer	Inner	Peri-nephric	Outer	Inner	Peri-nephric
Lauric	0·1	0·1	—	—	—	0·1	Trace	0·1	Trace
Myristic	0·9	0·8	0·9	0·9	0·6	0·8	0·9	0·9	0·9
Palmitic	26·5	27·5	29·3	26·0	29·4	31·3	30·1	30·7	30·3
Stearic	12·8	15·1	17·4	13·0	15·0	17·6	15·1	18·8	21·5
Tetradecenoic	0·2	0·2	0·3	0·2	0·2	0·1	0·2	0·2	0·2
Hexadecenoic	1·9	1·7	1·8	1·7	2·4	1·0	2·6	1·7	2·2
Oleic	46·8	44·2	40·3	45·6	40·0	38·8	39·5	37·2	34·1
Linoleic	7·9	7·3	8·1	9·1	9·6	8·3	8·2	7·1	7·3
Unsaturated C_{20-22}	2·9	3·1	1·9	3·5	2·8	2·0	3·4	3·3	3·5

(*b*) Molar % (excluding unsaponifiable matter)

Lauric	0·1	0·2	—	—	—	0·2	Trace	0·2	Trace
Myristic	1·0	0·9	1·0	1·1	0·7	0·9	1·0	1·1	1·0
Palmitic	28·4	29·5	31·3	27·9	31·4	33·3	32·2	32·7	32·4
Stearic	12·3	14·6	16·7	12·6	14·5	16·9	14·5	18·1	20·7
Tetradecenoic	0·2	0·2	0·4	0·2	0·2	0·1	0·3	0·3	0·2
Hexadecenoic	2·1	1·8	1·9	1·9	2·6	1·1	2·8	1·8	2·4
Oleic	45·6	42·9	39·1	44·4	38·8	37·6	38·2	36·0	33·1
Linoleic	7·7	7·2	7·9	8·9	9·4	8·1	8·0	6·9	7·1
Unsaturated C_{20-22}	2·6	2·7	1·7	3·0	2·4	1·8	3·0	2·9	3·1

Discussion

If we consider, in the first place, some of the more general data in Table 1, it will be seen that as fat is withdrawn from the depots to meet energy requirements the relative amount of tissue and water in the fatty tissue increases accordingly. The earlier stages of inanition show a preferential selection from the reserves of the outer back fat with a marked sparing of the more vital perinephric fat. Prolonged starvation, however, causes the largest degree of mobilization to be found in the inner back fat whereas the perinephric fat has been reduced in almost the same proportion as the outer back depots.

Preliminary investigation of possible changes in the nature of the depot fat, as far as can be judged from I.V. alone, seems to indicate that during the course of starvation the animal mobilizes its fat in a different way at later stages.

From Table 1 it can be seen that after 51 days' fasting each of the depots shows an increase in I.V. over those from the control, while after 135 days the reverse has happened—in all cases the depots are considerably more saturated in character than those in the control animal. There occurs then, at some stage of the fasting period a point of maximum I.V. (of the whole fats), after which the animal commences either to select preferentially, or (less probably) to saturate, unsaturated acids.

On the other hand, a preliminary examination of Table 2 shows that on the whole remarkably little difference in the proportions of component acids has accompanied prolonged starvation. In the first period of inanition the stearic acid content of all three depots shows little change whereas the other major saturated component acid (palmitic) has increased in the cases of the inner back and perinephric fat but shows a slight decrease in the outer back fat. Again, oleic acid has decreased in amount in each depot but there have been appreciable increases in linoleic and unsaturated C_{20-22} acids,[1] especially in the outer and inner back fat. The largest differences, taken all round, are to be found in the depots of the back, and this is in accordance with the previous observation, from Table 1, of the preferential mobilization of these reserves during the first period of starvation. It seems obvious that there has been some degree of selection of unsaturated fatty acids from the increased amount of linoleic acid in all depots. Limits in experimental technique forbid speculation as to changes occurring in the other minor component saturated and unsaturated acids except to say that in the second stage of starvation selection of fatty acids has produced a slight rise in the proportions of hexadecenoic acid.

The second stage of starvation has produced the following results common to all three depots: considerable mobilization of both unsaturated acids of the C_{18} series with parallel increases in the content of stearic acid; palmitic acid has found a common level at 32–33% (mol.); definite increases in the content of unsaturated C_{20-22} acids. We also find that after this drastic starvation the perinephric fat, judging from Table 2 alone, has been mobilized to the same extent as other reserves (an observation also confirmed by Table 1).

One must not lose sight of the fact that the units concerned in withdrawal of fat from the tissues are not individual fatty acids but triglyceride molecules; this will tend to diminish the spectacular nature of results in the event of preferential selection. Taking, as a case in point, the highly unsaturated acids of the C_{20} and C_{22} series present as minor components in pig depot fats, it is now recognized that no triglyceride molecule will contain more than one of these particular acyl groups (whilst in many others they will not be present at all). Let us suppose, by way of example, that these highly unsaturated C_{20-22} acids are preferentially selected; then, unless immediate resynthesis into glyceride takes place, the accompanying fatty acids will also be mobilized. Again, it should be remembered that the accompanying fatty acids will probably be a heterogeneous mixture of all the other acids present in the fat. Preselection of other acids may also occur and the picture of the process which is happening becomes extremely complex. Great caution is therefore necessary in interpreting the results given in Tables 1 and 2.

The experimental material at our disposal has furnished detailed statements of the component acids of the depot fats of pigs which have been fasted for very prolonged periods under conditions in which their metabolic processes were as little disturbed as possible. The lengthy periods of inanition should have per-

[1] The high proportion of this group of acids in the control animal is undoubtedly due to the high proportion, 15%, of fish meal and the daily dose of cod liver oil in the diet.

mitted the changes in the depot fats to develop more fully than otherwise; moreover, the loss of fat from the depots is considerably greater than in most other previous studies of this kind—the pig fasted for 135 days had lost nearly half its original live weight and over 70 % of its original back and perinephric fats. Nevertheless the most striking feature of the results is the relatively small change in composition of the fats in corresponding depots during the course of prolonged starvation; selective mobilization of certain fatty acids is to be observed, but this effect is subsidiary and of quite small magnitude.

By combining the data in Tables 1 and 2 it is possible to construct an approximate balance sheet of the total amount of each fatty acid actually present in each depot of the animals before and after starvation. The final results so obtained for the weights of each component acid present in each of the pig depot fats are shown in Table 3.

Table 3. *Total amounts of fatty acids in pig depots (g.)*

	Pig 175 (Control)			Pig 167 (51 days fasted)			Pig 173 (135 days fasted)		
Acid	Outer	Inner	Peri-nephric	Outer	Inner	Peri-nephric	Outer	Inner	Peri-nephric
Lauric	2	3	—	—	—	3	—	1	—
Myristic	22	26	30	13	13	20	6	7	9
Palmitic	641	907	990	374	616	796	202	227	295
Stearic	310	498	588	187	315	447	101	139	209
Tetradecenoic	5	7	10	3	4	3	1	1	2
Hexadecenoic	46	56	61	24	50	25	17	13	21
Oleic	1133	1458	1362	655	839	986	265	274	332
Linoleic	191	241	274	131	201	211	55	52	71
Unsaturated C_{20-22}	70	102	64	50	59	51	23	24	34
Total	2420	3298	3379	1437	2097	2542	670	738	973
Total saturated	975	1434	1608	574	944	1266	309	374	513
Total unsaturated	1445	1864	1771	863	1153	1276	361	364	460
Total C_{16}	687	963	1051	398	666	821	219	240	316
Total C_{18}	1634	2197	2224	973	1355	1644	421	465	612

Table 3 should be studied, not with respect to the actual amounts of acids present in the tissues, but in relation to the changes in amount of each acid produced during starvation. These changes become clearer if the % of each acid remaining at each of the two stages of fasting are calculated from Table 3, with the results given in Table 4.

Table 4 is confined to data for the major and more important minor component acids. In the case of the second period, i.e. 84 days, the figures represent the proportions of acids at the 51 days stage and not those present in the original control animal. Certain new facts come to light on examining this Table. In the first 51 days of starvation the amount of stearic acid remaining followed closely the proportion of total fatty acids which was unmobilized, whereas in the case of linoleic acid the proportion mobilized was considerably less for all three depots than the corresponding amounts for the total fatty acids. This can only be interpreted to mean that in the early stages of inanition there is considerable sparing of linoleic acid. Comparing figures for this acid and the corresponding total fatty acids in the depots for the subsequent 84 days of starvation we find that linoleic acid has not been spared but slightly preferentially metabolized. Right throughout the whole period of 135 days more unsaturated than saturated acids have disappeared and also acids of the C_{18} group

Table 4. *Percentage proportions of fatty acids remaining in pig depots after inanition*

	First 51 days			Subsequent 84 days		
Acid	Outer %	Inner %	Peri-nephric %	Outer %	Inner %	Peri-nephric %
Palmitic	58	68	80	54	37	37
Stearic	60	63	76	54	44	47
Hexadecenoic	52	89	41	71	26	84
Oleic	58	58	72	40	33	34
Linoleic	69	83	77	42	26	34
Unsaturated C_{20-22}	71	58	80	46	41	67
Total saturated	59	66	79	54	40	41
Total unsaturated	60	62	72	42	32	36
Total C_{16}	58	69	78	55	36	38
Total C_{18}	60	62	74	43	34	37
Total fatty acids	59	64	75	47	35	38

Note. This table should be interpreted in the following manner: The figures in each column represent the % of fatty acid unmobilized and should be compared with that at the foot of the Table. When the figure for an individual or group of fatty acids is higher than that at the foot, it is an indication that sparing has taken place; a lower figure therefore indicates preferential mobilization.

have been selected in preference to C_{16} acids; in connexion with this latter observation it will be seen that oleic acid, the most prominent component of pig fat, has suffered larger reductions than any other acid. There is marked sparing of the unsaturated C_{20} and C_{22} acids (although the data are slightly erratic); hexadecenoic acid shows similar sparing in some, but not all, cases. The results for these two groups of acids should however be treated with caution since experimental errors in the case of minor components, although certainly less than one unit, become a considerable percentage error when referred to the amount of acid found. In spite of this we can say with certainty that the C_{20-22} acids tend to remain in the fat reserves when animals are starved, although a fair proportion of them are metabolized. The unsaturated acids of the C_{20-22} series are of especial importance in relation to the keeping quality and palatability of pig fats, and it was hoped in the course of the present experiments to obtain information as to the mobilization of these acids. It was shown [Hilditch *et al.* 1939] that these are cumulatively absorbed, at all events up to a certain point, from fish oil or fish meal in the diet. From Tables 3 and 4 it will be seen that these acids have not decreased during inanition (especially in the later stages) in the same proportion as the other components.

Again, whilst oleic acid is mobilized to a marked degree during the early stages of starvation, the structurally closely related linoleic acid is withdrawn from the depots to a less extent than any other acid over the corresponding period. It may be that this observation is connected with the special functions which, as first shown by Burr & Burr [1929], linoleic acid exercises in the animal diet. On the other hand, it is equally likely that the sparing of linoleic and of the unsaturated C_{20} and C_{22} acids during fat mobilization may be directly connected with the fact [Hilditch *et al.* 1939] that both of these acids arrive in pig depot fats by assimilation from dietary fats and are not synthesized by the animal; and that, correspondingly, they are less readily mobilized than those acids which are synthesized by the animal from carbohydrate.

To compare the depot fats of the pig and of fish is perhaps unprofitable, but the present results nevertheless resemble those of Lovern [1938] on flesh fats of

fasting herrings in that in both cases there is preferential utilization of unsaturated components; this effect, however, was little in evidence in the case of salmon during their fasting period [Lovern, 1934]. Similarities between our present data on pig depot fats and those of Longenecker [1939, 1, 2, 3] on rat depot fats are not very marked; the chief resemblances are the decrease in total C_{16} acids observed in two of the pig depots during the first 51 days of inanition. On the other hand, in one experiment with rats, Longenecker observed an increase in oleic acid content, and a decrease in linoleic acid content, during mobilization of fat from the rat depots.

The general results of this study are that, apart from relatively minor differences, there is no great evidence of selectivity in the mobilization of any one fatty acid component of the depot fats during starvation of the pig; the two most prominent subsidiary effects are the preferential removal of oleic acid during the later stages of inanition, and the definite reluctance in the earlier stages to mobilize those acids (linoleic and unsaturated C_{20-22} acids) which are derived from ingested dietary fats. Here, again, it should once more be emphasized that the problem of fat mobilization cannot be satisfactorily dealt with in terms of the fatty acids, but of the mixed triglycerides in which they are present. The most abundant glycerides in pig depot fats are palmitodioleins and oleopalmitostearins. If oleic acid is selectively desired, it therefore involves the concurrent removal of 1 mol. of palmitic acid for every 2 mol. of oleic acid, or of 1 mol. each of palmitic and stearic acid as well as oleic acid, in these respective glycerides. Similarly, as has already been pointed out, any of the minor component acids will only be represented once in the three acyl groups of any triglyceride and therefore, although an unsaturated C_{20} acid, for example, may not be so readily dealt with as oleic acid, a triglyceride molecule containing two oleic and one unsaturated C_{20} acyl group might well be attacked for the sake, as it were, of its oleic acid content. The problem of fat mobilization is, in fact, essentially one from which considerations of glyceride structure cannot be excluded: the individual mixed triglycerides present in the depot fat clearly have a profound bearing on the process.

Lastly, it should be borne in mind that, although the amounts of each acid combined in the depot fats have decreased during starvation of the pigs, concurrent synthesis of glycerides from non-fatty or fatty body constituents of the animal is not precluded, even though it is unlikely to take place to any great extent under the condition of fasting. The possibility cannot be excluded that, simultaneously with mobilization, building up (to a minor extent) of glycerides either from newly formed fatty acids or from acid fragments of partly metabolized mixed glycerides takes place. Well-known recent work by several investigators on the ingestion and mobilization of "labelled" fats has shown that both deposition and removal of fats in animal depots are relatively rapid and probably concurrent processes. The adipose tissues are now clearly recognized as dynamic, rather than static, reservoirs.

We again offer our thanks to Dr J. Hammond for his courtesy in providing the experimental material, to Drs E. H. Callow and C. H. Lea for their assistance in the extraction of the fats, and to the Department of Scientific and Industrial Research for permission to publish our results.

REFERENCES

Burr & Burr (1929). *J. biol. Chem.* **82**, 345.
Dowell (1920). *Ann. Rep. Oklahoma Agric. Exp. Sta.* **29**, 28.
Frank (1926). *Mschr. Kinderheilk.* **31**, 265.
Hilditch, Lea & Pedelty (1939). *Biochem. J.* **33**, 493.
Longenecker (1937). *J. Soc. chem. Ind., Lond.*, **56**, 199*T*.
—— (1939, 1). *J. biol. Chem.* **128**, 645.
—— (1939, 2). *J. biol. Chem.* **129**, 13.
—— (1939, 3). *J. biol. Chem.* **130**, 167.
Lovern (1934). *Biochem. J.* **28**, 1955.
—— (1938). *Biochem. J.* **32**, 676.
Mann (1936). *Fette u. Seifen*, **43**, 155.
Stefko (1928). *Biochem. Z.* **195**, 396.

8. STUDIES IN DETOXICATION

4. THE RESOLUTION OF *dl-ISO*MENTHOL THROUGH ITS CONJUGATION WITH GLUCURONIC ACID IN THE RABBIT

By R. TECWYN WILLIAMS

From the Physiology Department, Medical School, Hospitals Centre, Birmingham

(*Received 20 November 1939*)

It has been shown by Williams [1939] that *dl*-menthol could be resolved to give optically pure *d*-menthol by making use of the glucuronic acid detoxication mechanism of the rabbit. In a similar manner *dl-iso*menthol can be resolved to give optically pure *d-iso*menthol. *l-iso*Menthol can also be obtained but the most laevorotatory fraction was no more than 84 % optically pure. The resolution of *dl-iso*menthol is of special interest in that it has not been done before. Chemical methods, which can be used for the resolution of *dl*-menthol, fail when applied to *dl-iso*menthol mainly because of mixed crystal formation. Read *et al.* [1933] state, "No optical resolution of *dl-iso*menthol has yet been recorded and we now show that neither of the methods recently devised for the resolution of *dl*-menthol [Read & Grubb, 1931; 1933] is applicable to *dl-iso*-menthol".

When *dl-iso*menthol is fed to rabbits, 55 % of it is excreted in the urine conjugated with glucuronic acid; the *iso*menthylglucuronide excreted contains about 60 % *d*- and 40 % *l*-isomeride [Williams, 1938]. The mixed *iso*menthyl-glucuronides were isolated as NH_4 salts. It is this mixture which I have used in the work described here. The mixture, on crystallization from water, deposited homogeneous plates which proved to be *ammonium* dl-iso*menthyl*-d-*glucuronate* ($[\alpha]_D - 56°$ in water). Repeated recrystallization of this salt failed to alter its rotation and it gave *dl-iso*menthol on acid hydrolysis. This salt appears to be a "partially racemic" compound of the Ladenburg type (see Cohen [1924] and compare the (−)menthyl *r*-mandelate of McKenzie [1904]). Acidification of solutions of this salt did not give the *dl*-acid, but an amorphous solid which was mainly impure *d-iso*menthylglucuronide. Two recrystallizations of this solid from water gave pure *d-iso*menthylglucuronide, which yielded *d-iso*menthol quantitatively on hydrolysis. The filtrate from the above amorphous solid deposited fine needles on further acidification. Various fractions of these needles were collected showing rotations varying from $[\alpha]_D - 59{\cdot}7°$ to $-65{\cdot}2°$ (in alcohol). Recrystallization from water produced only small changes in rotation. The fraction $[\alpha]_D - 65{\cdot}2°$ gave, on hydrolysis, an *iso*menthol which contained 66 % of the *l*-form. No fraction was obtained which gave a more pure *l-iso*menthol than this. It seems probable that we have here an example of mixed crystal formation. The filtrates after the separation of the needles were therefore extracted with ether. Removal of the ether gave a stiff syrup which, when dissolved in water, failed to deposit any crystalline material. The syrup was therefore hydrolysed and the *iso*menthol obtained contained 84 % of the *l*-form ($[\alpha]_D - 17{\cdot}6°$ in alcohol). The yield of optically pure *d-iso*menthol obtained by this method did not exceed 5 % of the *dl-iso*menthol fed to the rabbits.

Experimental

Preparation of ammonium dl-iso*menthylglucuronate*

From 15 g. *dl-iso*menthol fed to rabbits, 12 g. of crude ammonium *iso*-menthylglucuronate were isolated from their urine by the method already described for the isolation of the menthyl derivative [Williams, 1939]. This salt on recrystallization from water gave *ammonium* dl-iso*menthylglucuronate* as elongated plates (yield 8 g.). The crude filtrate from this salt was not further investigated. The salt was purified by recrystallization from water. The pure salt showed $[\alpha]_D^{28°} -56°$ in water ($c=1$) which was not altered on recrystallization (Found: C, 51·0; H, 9·0; N, 3·5; H_2O, 7·4 %. $(C_{16}H_{27}O_7NH_4)_2$, $3H_2O$ requires C, 51·0; H, 9·1; N, 3·7; H_2O, 7·2 %). On hydrolysis with dilute H_2SO_4 this salt gave *dl-iso*menthol, M.P. 53–54°[1] and mixed M.P. 54° and $[\alpha]_D^{25°}$ 0° ± 1° in alcohol ($c=1·1$ and 2; two different specimens). Attempts to resolve this salt by fractional precipitation of its aqueous solution with ammonium sulphate failed to give any appreciable separation.

The separation of d-iso*menthylglucuronide*

The ammonium *dl*-salt (7 g.) was dissolved in 90 ml. of water at room temperature and 10 ml. of $2N$ H_2SO_4 added. After 4 hr., a white translucent waxy solid had separated. This was collected and on drying it gave a hard amorphous mass (2·9 g.) showing $[\alpha]_D -45·5°$ in alcohol. The rotation of this substance was consistently between −45° and −46° in all the experiments carried out. Two recrystallizations of this substance from 150 and 100 ml. of water respectively, gave pure *d-iso*menthylglucuronide (1·21 g.) as long fine needles, M.P. 125° (mixed M.P. 125–126° with an authentic specimen) and showing $[\alpha]_D -43°$ in alcohol ($c=1$) (Williams [1938] gives $[\alpha]_D -43·3°$ in alcohol and M.P. 126°). A further 0·145 g. was obtained from the filtrates at various stages, making the total yield of *d-iso*menthylglucuronide 1·355 g.

The filtrate from the waxy solid above was now treated with 10 ml. of $2N$ H_2SO_4 and kept 24 hr. at 0°. The solution was filled with long fine delicate needles (2·2 g.), M.P. 115° and showing $[\alpha]_D^{21°} -61·5°$ in alcohol. These needles, on repeated recrystallization from water acidified with H_2SO_4 showed only small increases in laevorotation. From another resolution, needles showing $[\alpha]_D^{27°} -65·2°$ in alcohol ($c=1$) were obtained and this figure was the maximum laevorotation obtained in all the resolutions carried out for this work. The latter material gave on hydrolysis an *iso*menthol, M.P. 60° and showing $[\alpha]_D^{28°} -9·1°$ in alcohol ($c=1$). It therefore contained 67 % *l-iso*menthol.

The filtrate from the above needles was further acidified with 10 ml. of $2N$ H_2SO_4, but even after standing a week the solution deposited no more crystals. The filtrate was therefore extracted with ether to remove any *iso*-menthylglucuronide remaining in the solution. The ethereal extract, after drying with anhydrous Na_2SO_4, was evaporated and about 1 g. of a stiff syrup, which eventually set to a glass, was obtained. This syrup yielded no crystalline material after dissolution in water and acidifying. The acidified solution was therefore steam-distilled and 0·4 g. of optically impure *l-iso*menthol (84 % *l*-) was obtained which had M.P. 74–76° and $[\alpha]_D -17·6°$ in alcohol ($c=2$).

[1] All melting points are uncorrected.

Preparation and identification of d-iso*menthol from the glucuronide*

*d-iso*Menthylglucuronide (2 g.) obtained from the above resolutions was hydrolysed with 20 ml. 0·5*N* H_2SO_4, the *iso*menthol formed being recovered by a simultaneous steam distillation. The distillate containing the solid *iso*menthol was extracted with light petroleum. The extract on evaporation gave optically pure *d-iso*menthol (0·85 g.) in almost theoretical yield. It formed long needles M.P. and mixed M.P. 82–83° and showed $[\alpha]_D^{21°}+25·3°$ in alcohol ($c=2$). Read & Grubb [1934] give $[\alpha]_D+25·9°$ and M.P. 82·5°. It was further identified as its 3:5-dinitrobenzoate, which formed long needles M.P. 147° and had mixed M.P. 146° with an authentic specimen, M.P. 145° (Read *et al.* [1933] give M.P. 145°).

Summary

*dl-iso*Menthol has been resolved for the first time, through the *iso*menthylglucuronide synthesized in the rabbit, following oral administration of *dl-iso*menthol.

The crude ammonium *iso*menthylglucuronate isolated from the rabbit urine gave, on recrystallization from water, *ammonium* dl-iso*menthylglucuronate* which appears to be a partially racemic compound of the Ladenburg type. This salt cannot be resolved as such, but on conversion into the free acid, crude *d-iso*menthylglucuronide was formed as a waxy solid. The crude material on recrystallization from water gave the pure crystalline acid identical with an authentic specimen. The glucuronide yielded optically pure *d-iso*menthol on hydrolysis. *l-iso*Menthol was not isolated in optically pure form, although material of 84 % optical purity was obtained.

REFERENCES

Cohen (1924). Organic Chemistry, **2**, 219. London: Arnold.
McKenzie (1904). *J. chem. Soc.* **85**, 378, 1249.
Read & Grubb (1931). *J. chem. Soc.* p. 188.
—— —— (1933). *J. Soc. chem. Ind.* **51**, 329 *T*.
—— —— (1934). *J. chem. Soc.* p. 313.
—— —— & Malcolm (1933). *J. chem. Soc.* p. 171.
Williams (1938). *Biochem. J.* **32**, 1849.
—— (1939). *Biochem. J.* **33**, 1519.

9. THE INACTIVATION OF OESTRONE

By W. W. WESTERFELD[1]

From the Department of Biochemistry, Oxford

(*Received 21 November 1939*)

The work of a large number of investigators has demonstrated quite conclusively that the administration of large amounts of oestrogens does not lead to the excretion of appreciable amounts of these substances in the urine or faeces. The actual amounts that can be recovered vary from 3 to 20 % of the quantity administered, and depend upon the hydrolysis of the conjugated oestrogens before the extraction and testing of the urine. The maximum excretion of the oestrogen occurs within the first 24 hr. of its administration, and practically ceases after 48–96 hr. irrespective of the amount administered [Mazer & Israel, 1937]. The failure of the administered oestrogen to be excreted is not due to its storage in the body [Zondek, 1934; Dingemanse & Laqueur, 1937; Frank *et al.* 1932].

From these and similar studies there is no doubt that only a small portion of the oestrogen is excreted, and that the remainder is rapidly destroyed or altered (beyond simple conjugation) so that it can no longer be detected by the usual means. It is generally believed that this inactivation is brought about by the liver [Zondek, 1934; Israel *et al.* 1937].

The nature of this change is unknown, and the closely related compounds that are known have failed to indicate the course of this reaction. The presence of the more unsaturated oestrogens, equilin and equilenin, in the urine of the pregnant mare suggests these compounds as possible metabolites in this species, but it is improbable that equilenin occurs in human urine [Marrian, 1938; Marx & Sobotka, 1938]. Similarly, the presence of oestriol in human pregnancy urine might be construed as indicating a metabolic pathway for oestrone, but the inability of the animal to convert oestrone into oestriol except under the influence of progesterone [Smith *et al.* 1938] would eliminate this change as an important pathway for inactivation. The completely saturated hexahydro-oestradiols, discovered in the urine of non-pregnant females [Marker *et al.* 1938, 1, 2], are undoubtedly derived from the natural oestrogens, and it has been suggested that they are formed by non-enzymic reductive processes in the course of the utilization of oestrone as a hormone. The exact role of these derivatives in the "inactivation" process, if any, is unknown.

It is significant that all the natural oestrogens are phenols, and the presence of an active hydroxyl group in such a molecule would present a likely point of attack for oxidation by the body. The first stage in such an oxidation would be the introduction of a second hydroxyl group in the *o*- or *p*-position. The belief that this is the method utilized by the body for the inactivation of the oestrogens is supported by the following: (1) phenols in general are metabolized in this manner; (2) H_2O_2 will readily inactivate the oestrogens, and its characteristic action on monohydric phenols is the oxidation of these substances to *o*- or *p*-dihydric phenols; (3) the enzyme tyrosinase is capable of oxidizing many

[1] Fellow in the Medical Sciences of the National Research Council.

monohydric phenols to *o*-dihydric phenols (and subsequently to *o*-quinones), and is also capable of inactivating the oestrogens.

A few studies of the polycyclic compounds have indicated that like benzene and the simple phenols they too may be converted into mono- and di-hydric phenols. Naphthalene is oxidized by the body in a manner similar to that of benzene inasmuch as it is partially converted into α-naphthol [Rossbach, 1884], and this in turn is oxidized to a dihydroxy-naphthol [Lesnik & Nencki, 1886]. An excretion product of anthracene has been identified [Boyland & Levi, 1935; 1936] as a dihydroxy-dihydroanthracene. A phenanthrol glycuronic acid of unknown constitution was found in the urine after the administration of phenanthrene [Bergell & Pschorr, 1903].

The introduction of one or more hydroxyl groups into the benzene nucleus by the body seems to be a well-defined metabolic pathway. This is true not only of benzene, naphthalene, anthracene, phenanthrene and phenylalanine as indicated above, but is characteristic of the synthetic oestrogen stilbene [Stroud, 1939] and all aromatic substances in general [Dakin, 1922]. It is not certain that an aromatic character is essential for this reaction, for practically nothing is known of the metabolism of the more saturated ring compounds. The production of mono- and di-hydroxy compounds from decahydronaphthalene and tetrahydronaphthalene [Pohl & Rawicz, 1919; Bernhard, 1939] would indicate that it is not necessary, but the point needs further study. Its importance in a study of the metabolism of the steroids is obvious.

Since all of the natural oestrogens are phenols, it is quite conceivable that they are metabolized by the body in a similar manner, and that the resulting dihydroxy compounds are physiologically inactive.

Metabolism of oestrone methyl ether

An attempt was made to prevent the inactivation of oestrone by the body by blocking the phenolic group. Recovery experiments were carried out with oestrone methyl ether by A. Palmer[1] [personal communication], who found that the inactivation was not prevented and that the substance was probably demethylated by the body into free oestrone. The following method was employed. 1·0 mg. of the methyl ether of oestrone (M.P. 170–171°) was injected daily for 7 days into a castrated monkey, and the corresponding urine samples were collected. These were pooled and extracted for the free and combined oestrogens; each extract was further separated into neutral and phenolic fractions, and then assayed in spayed mice. The total activity recovered was equivalent to about 100 μg. oestrone, of which one-third was free and two-thirds combined. Nearly all of the activity, whether free or combined, was found in the phenolic fraction. This suggests that the compound was demethylated by the body, possibly as a prerequisite to its oestrogenic action.

Action of H_2O_2 on phenols

Dakin & Herter [1907] have emphasized the similarity between the reactions carried out by the body and those effected through oxidation with H_2O_2. In the case of the phenols this is borne out to a striking degree, especially in so far as the introduction of a second hydroxyl group is concerned.

Benzene has been oxidized with H_2O_2 to phenol [Leeds, 1881], and that in turn oxidized to quinol and catechol [Martinon, 1885; Henderson & Boyd, 1910]. The cresols are oxidized to the corresponding diphenols [Henderson & Boyd,

[1] The author wishes to express his thanks to Dr Allen Palmer for permission to quote these results.

1910], i.e. *o*-cresol to toluquinol (and some toluquinone), *m*-cresol to toluquinonorcin, and *p*-cresol to homocatechol. 3:5-Diethylphenol yields 3:5-diethyl *p*-benzoquinone. Thymol is converted into thymoquinone and tetrahydroxycymol and carvacrol are oxidized like thymol [Henderson & Boyd, 1910].

The ability of H_2O_2 to introduce hydroxyl groups into the aromatic nucleus has also been demonstrated for benzoic acid [Dakin & Herter, 1907]. The first product formed is the *o*-, *m*- or *p*-hydroxy benzoic acid, and further oxidation leads to the introduction of a second hydroxyl group in the *o*-position to the first, i.e. 2:3- or 3:4-dihydroxybenzoic acid.

The oxidation of phenylalanine by H_2O_2 is identical with the first steps in its oxidation by the body. Thus it gives rise to tyrosine and then to 3:4-dihydroxyphenylalanine [Raper, 1932]. Similarly tyramine yields 3:4-dihydroxyphenylethylamine.

The oxidation of cholesterol with benzoyl peroxide or selenium dioxide results in the formation of Δ^5-cholestene-diol-3:4 [Rosenheim & Starling, 1933]. This "oxycholesterol" was studied extensively by Lifschutz, who believed it to be a normal metabolic product of cholesterol. 2:3-Dihydroxyandrostane derivatives have been prepared from Δ^2-androstene compounds by treatment with H_2O_2, and the same type of reaction is obtained with Δ^2-cholestene [Marker & Plambeck, 1939].

Action of H_2O_2 on oestrone

The fact that H_2O_2 destroys the activity of the oestrogens has been known for some time [Thayer *et al.* 1928], but the nature of the reaction involved has never been determined. In view of the preceding studies it is likely that the course of this oxidation is similar to that of these other well-defined cases. Some evidence has been obtained to indicate that the attack is on the phenolic portion of the molecule, and the isolation of the inactivated products is now in progress.

Oestrone is not readily inactivated by H_2O_2 in a neutral solution. 100 mg. oestrone were dissolved in 20 ml. acetone and treated with a large excess of H_2O_2 by successive additions over a period of 1 month. The solution was refluxed after some of the additions and allowed to stand at room temperature after other additions, but at the termination of the experiment over 50 % of the oestrone was recovered unchanged and as active as it was originally. On the other hand, when the oestrone was dissolved in NaOH it was rapidly and completely inactivated by H_2O_2 at room temperature.

The inactivation by peroxide in alkaline solution was prevented by blocking the phenolic group. 5 mg. of the methyl ether of oestrone (M.P. 170–171°) were dissolved in 100 ml. alcohol, and the solution was divided into two equal portions, one of which served as a control. To the other, 40 ml. of H_2O_2 (2400 mg.) and 10 ml. of 20 % NaOH were added, and the solution was allowed to stand at room temperature for 5 days. Under these conditions a much larger quantity of free oestrone would have been completely destroyed, but the activity of the methyl ether was practically unaffected. Excess peroxide was removed by catalase, and the oestrogen was recovered by extraction with ether. Both the control and experimental solutions were taken up in oil and assayed simultaneously in spayed mice. There was no appreciable difference between the activities of the two solutions, each containing about 800 mouse units. This is similar to earlier experience in which blocking the phenolic group prevented oxidation by benzoyl peroxide [Butenandt *et al.* 1932].

Action of tyrosinase on phenols

The general action of tyrosinase on monohydric phenols is to introduce a second hydroxyl group into the *o*-position to the one already present and then to oxidize the dihydric phenol to an *o*-quinone [Raper, 1928]. The fact that *o*-quinones are produced from phenols was pointed out by Szent-Györgyi [1925], and has been confirmed by the isolation of their anilino derivatives [Pugh & Raper, 1927]. The anilino derivative of *o*-benzoquinone has been obtained from phenol and catechol, and the corresponding derivative of homoquinone has been obtained from *m*-cresol, *p*-cresol and homocatechol. The *m*- and *p*-methoxyhydroxybenzenes act like the corresponding cresols in producing *o*-quinones, and these have been isolated as the anilino-quinones. The same type of oxidation is produced in the initial stages of the formation of melanin from tyrosine [Raper, 1928].

Although *o*-quinones are undoubtedly produced in the oxidation of phenols, they are probably not the end products of the reaction [Pugh & Raper, 1927; Nobutani, 1936]. Wagreich & Nelson [1936] have suggested that another hydroxyl group is introduced into the *o*-quinone.

The action of tyrosinase on phenols is specific to the extent that it generally attacks those phenols that give rise to catechol derivatives and quinones, but has no action on those substances that form quinols [Pugh & Raper, 1927; Onslow & Robinson, 1925; 1926; 1928]. It will catalyse the oxidation of a large number of monophenols, as tyrosine, phenol, *m*- and *p*-cresol, *m*- and *p*-methoxyhydroxybenzene, *p*-hydroxyphenylethyl alcohol, *p*-aminophenol, *p*-chlorophenol etc., to *o*-dihydric phenols. In each case this is the first step in the oxidation regardless of the course of further changes.

Action of tyrosinase on the oestrogens

The experimental portion of this report has shown that tyrosinase also attacks the oestrogens, rendering them inactive. From the preceding discussion it is relatively certain that the first stage in this oxidation would be the production of an *o*-dihydric phenol, though further oxidation is also likely.

The inactivation by tyrosinase appears to be a true enzymic action and not merely an artefact. The enzymic effect is abolished by preliminary boiling of the tyrosinase, and it cannot be duplicated by a system of catechol oxidase plus catechol. The inactivation is not due to secondary production of H_2O_2 because it also takes place in the presence of catalase.

Experimental

Each oestrogen was made up in a phosphate buffer by dissolving it in the correct amount of NaOH (14–16 ml. of 20% diluted to 300 ml., the exact amount being determined beforehand), warming and adding 13·622 g. KH_2PO_4. The salt was dissolved with an additional quantity of water, and the solution was then cooled and diluted to 1000 ml. The amount of NaOH added was arranged to give the final solution a *p*H of 7·35 so that it could also be used in tissue respiration studies. The solution was stable, and lost none of its activity upon standing. As a routine it was filtered before using, and its activity determined by assay.

The tyrosinase extract was made up for each experiment from a stable preparation of mushrooms. This preparation was obtained by mincing the mushrooms into ice-cold alcohol, squeezing the alcohol through muslin, and

washing several times with fresh portions of cold alcohol. This extraction with alcohol had the two-fold objective of dehydration and removal of most of the naturally occurring phenols. The remaining alcohol was removed by pressure as far as possible from the mushroom residue, and the residue was rapidly dried *in vacuo*. It was then ground to a fine powder. The tyrosinase extract was prepared from this powder by extracting each gram with 20 ml. of 0·1 % NH_4OH. The solution was squeezed through muslin and centrifuged, and the supernatant fluid was decanted and tested for its activity on *p*-cresol before applying it to the oestrogen solution.

The inactivation of the oestrogen was carried out by treating 50 ml. of the solution with 15 ml. of the tyrosinase extract. After adjusting to *p*H 8 (approx.) with dilute NaOH, it was incubated for 5 hr. at 38°. During the incubation it was shaken vigorously to keep the solution adequately aerated. The enzymic action was stopped by placing the solution in boiling water for ½ hr.; sufficient NaOH was added to make the *p*H 9, and the solution was diluted to a known volume for assay in spayed rats or mice by the technique of Kahnt & Doisy [1928].

The inactivation was carried out at *p*H 8 because the greatest destruction of tyrosine by tyrosinase occurs at this *p*H [Evans & Raper, 1937]. The results are summarized in Table 1. Both oestrone and oestradiol lose at least 75 % of their

Table 1. *The effect of treatment with various enzymes, etc., on the activity of the oestrogens*

Oestrogen	Nature of treatment: incubation with	Activity of solution: Before treatment	Activity of solution: After treatment
Oestrone	Tyrosinase	450 m.u.	100 m.u.
	Boiled tyrosinase	450	450
	Fe	450	450
	Cu	450	450
	Catechol oxidase	450	375
	Catechol oxidase plus catechol	450	375
	H_2O_2	450	<100
	Catalase	450	400
	Catalase plus H_2O_2	450	400
	Catalase plus tyrosinase	450	150
Oestradiol	Tyrosinase	250 r.u.	<60 r.u.
	Boiled tyrosinase	250	250
	Fe	250	250
	Cu	250	250
	H_2O_2	250	<60
	Catalase plus H_2O_2	450	400
	Catalase plus tyrosinase	450	100
Diethylstilboestrol	Tyrosinase	600 r.u.	100 r.u.

original activity by this treatment with tyrosinase; the synthetic oestrogen diethylstilboestrol is inactivated to an even greater degree. In 7 out of 15 such experiments with oestrone there was no sign of oestrogenic activity in any one of a group of 8 to 25 mice when the final solution was tested at the level of 100 mouse units; in these cases the inactivation must have been much greater than 75 % since both the original and control solutions contained 450 mouse units. In the other 8 experiments with oestrone the final solutions contained very nearly 100 mouse units. A few experiments carried out at *p*H values between 7 and 8 indicated that the inactivation was practically as great at any point in this range.

A control on the enzymic nature of this reaction was made by testing a boiled extract of tyrosinase. Both the experimental and control tests were

LINDA HALL LIBRARY
KANSAS CITY, MO.

carried out simultaneously and identically except that in the latter case the 15 ml. portion of the tyrosinase extract was placed in boiling water for ½ hr. before being added to the oestrogen solution. The controls were assayed simultaneously with the experimental solutions, and showed no loss of activity.

Several tests on the effect of inorganic salts were also made. 50 mg. of Fe or Cu, as $FeCl_3$ or $CuSO_4$, were added to the oestrogen solution and incubated in the usual way. Assays showed there was no loss of activity in either case, but the results were somewhat complicated by the precipitation of these ions in the buffer. The oxidation of monophenols is generally not catalysed by inorganic ions, but catechol derivatives may be affected [Bhagvat & Richter, 1938].

Onslow & Robinson [1928] have suggested that the oxidation of monophenols by tyrosinase is not a straightforward enzymic action, but is a secondary reaction caused by the *o*-quinone, H_2O_2, or organic peroxide produced by the action of a polyphenolase on a catechol derivative present in the crude extract. The change from catechol to *o*-quinone would be reversible, and hence only a small amount of a catechol derivate in the extract would be necessary for this secondary catalysis. Catechol derivatives occur naturally in most of the tyrosinase sources, and it is known that *o*-quinones are capable of effecting some secondary oxidations.

The essence of this theory has been supported by Richter [1934], but the idea that the *o*-quinone was solely responsible for the secondary oxidations was abandoned [Onslow, 1931]. The recent isolation [Dalton & Nelson, 1938; 1939] of a crystalline tyrosinase capable of catalysing the oxidation of both mono- and di-hydric phenols seems to have rendered this theory superfluous. Nevertheless it was possible that the inactivation of the oestrogens by a crude tyrosinase extract could have been accounted for on such a basis.

This point was tested by determining the effect of catechol oxidase plus catechol on oestrone under the same conditions in which the oestrone had been inactivated by tyrosinase. The preparation of catechol oxidase[1] was approximately 20 % pure and was intensely active towards catechol but practically inert with *p*-cresol. The catechol oxidase itself had very little effect upon the activity of the oestrone solution, and there was no greater effect when 25 mg. of catechol were added to the reaction mixture (Table 1). The oxidation of the catechol by the enzyme was indicated by the immediate and intense colour produced, but the oxidation products of the catechol were not capable of secondarily oxidizing the oestrone.

A certain amount of evidence has been obtained to indicate that H_2O_2 or an organic peroxide is produced in the reaction between tyrosinase and a phenol. It has been rather difficult to demonstrate conclusively that H_2O_2 is produced, and though it has been questioned [Nobutani, 1936; Sutter, 1936] it remains a possibility [Onslow & Robinson, 1926; Evans & Raper, 1937]. The peroxide that is produced would be a by-product of the reaction, and while the enzyme peroxidase may [Evans & Raper, 1937] or may not [Richter, 1934; Szent-Györgyi, 1925] be involved in the subsequent dissipation of the peroxide, it could possibly effect some secondary oxidations.

Thus, if the extract of tyrosinase used in these experiments contained any naturally occurring phenols (a point guarded against by the extraction of the mushrooms with alcohol), it might also contain some H_2O_2, and the peroxide would inactivate the oestrogen directly. Under the conditions of these experiments H_2O_2

[1] The author wishes to express his thanks to Dr Keilin for supplying (through Mrs Philpot) the catechol oxidase.

will completely inactivate the oestrogens. This was confirmed by adding 15 mg. H_2O_2 to 50 ml. of the oestrogen solution at *p*H 8, incubating and treating the solution in the same way that the tyrosinase experiments were conducted.

The inactivation of the oestrogens by H_2O_2 could be prevented by adding catalase to the oestrogen solution before introducing the peroxide. A highly active catalase, prepared from liver by the method of Zeile [1931], itself had no effect on the activity of the oestrogen solution under these standardized conditions, and when added together with 60 mg. H_2O_2 it destroyed the peroxide before the latter could inactivate the oestrogen (Table 1).

The addition of catalase to the oestrogen solution before its incubation with tyrosinase did not prevent the enzymic inactivation of the oestrogen. The experiments were conducted as before and any H_2O_2 present in the extract or produced during the incubation would have been destroyed. The inactivation which took place under these conditions must have been a genuine enzymic effect, and while it was not quite as extensive as that which occurred in the absence of catalase, there was no doubt as to the essential feature of the reaction.

Discussion

The ultimate proof of the method whereby the oestrogens are inactivated by the body lies in the isolation of the inactive products from the excreta of a suitable subject. In the absence of this more conclusive proof the evidence herein presented can do little more than suggest a likely pathway. But it is significant that each of the analogies has indicated the same pathway.

The introduction into the oestrone molecule of another hydroxyl group in the *o*- or *p*-position could theoretically produce three compounds; *o*-substitution would give rise to either 2:3- or 3:4-dihydroxy-17-keto-oestratriene; *p*-substitution would yield 10-hydroxy-3:17-diketo-oestradiene(1:4). These derivatives of oestrone are unknown at present. In the case of the synthetic oestrogens the substitution of any group directly into the phenolic nucleus greatly decreases its activity and may inactivate it entirely. Similar derivatives of the natural oestrogens have not been described, except for the halogen derivatives which are relatively inactive [Thayer *et al.* 1931].

Summary

1. Attention is called to the fact that the oestrogens may be metabolized by the body similarly to other phenols, which are oxidized to *o*- or *p*-dihydric phenols. The same type of oxidation of phenols is brought about *in vitro* by H_2O_2, and *o*-dihydric phenols are also produced as the first step in the oxidation of monophenols by tyrosinase.

2. Oestrone was readily inactivated by H_2O_2 in alkaline solution but not in neutral solution; the methyl ether of oestrone was not inactivated by H_2O_2 in alkaline solution.

3. Oestrone, oestradiol, and diethylstilboestrol were inactivated by incubation with tyrosinase; a boiled tyrosinase extract had no action.

4. Incubation with catechol oxidase or a combination of catechol oxidase plus catechol did not produce any appreciable inactivation.

5. Complete inactivation resulted from the addition of H_2O_2, but could be prevented by the simultaneous addition of catalase; however, the addition of catalase did not prevent the inactivation by tyrosinase.

The author is greatly indebted to Prof. R. A. Peters for many suggestions and facilities in carrying out these studies.

REFERENCES

Bergell & Pschorr (1903). *Hoppe-Seyl. Z.* **38**, 16.
Bernhard (1939). *Hoppe-Seyl. Z.* **257**, 54.
Bhagvat & Richter (1938). *Biochem. J.* **32**, 1397.
Boyland & Levi (1935). *Biochem. J.* **29**, 2679.
—— —— (1936). *Biochem. J.* **30**, 728.
Butenandt, Stormer & Westphal (1932). *Hoppe-Seyl. Z.* **208**, 149.
Dakin (1922). Oxidations and Reductions in the Animal Body. London: Longmans Green and Co.
—— & Herter (1907). *J. biol. Chem.* **3**, 419.
Dalton & Nelson (1938). *J. Amer. Chem. Soc.* **60**, 3085.
—— —— (1939). *J. Amer. Chem. Soc.* **61**, 2946.
Dingemanse & Laqueur (1937). *Amer. J. Obstet. and Gynaec.* **33**, 1000.
Evans & Raper (1937). *Biochem. J.* **31**, 2155.
Frank, Goldberger & Spielman (1932). *Proc. Soc. Exp. Biol., N.Y.*, **29**, 1229.
Henderson & Boyd (1910). *J. chem. Soc.* **97**, 1659.
Israel, Meranze & Johnston (1937). *Amer. J. med. Sci.* **194**, 835.
Kahnt & Doisy (1928). *Endocrinology*, **12**, 760.
Leeds (1881). *Ber. dtsch. chem. Ges.* **14**, 975.
Lesnik & Nencki (1886). *Ber. dtsch. chem. Ges.* **19**, 1534.
Marker & Plambeck (1939). *J. Amer. chem. Soc.* **61**, 1332.
—— Rohrmann, Lawson & Wittle (1938, 1). *J. Amer. chem. Soc.* **60**, 1901.
—— —— Wittle & Lawson (1938, 2). *J. Amer. chem. Soc.* **60**, 1512.
Marrian (1938). *Ergebn. Vitamin Hormon Forsch.* **1**, 419.
Martinon (1885). *Bull. Soc. Chim.* **43**, 155.
Marx & Sobotka (1938). *J. biol. Chem.* **124**, 693.
Mazer & Israel (1937). *J. Amer. med. Ass.* **108**, 163.
Nobutani (1936). *J. Biochem.* **23**, 455.
Onslow (1931). Principles of Plant Biochemistry. Camb. Univ. Press.
—— & Robinson (1925). *Biochem. J.* **19**, 420.
—— —— (1926). *Biochem. J.* **20**, 1138.
—— —— (1928). *Biochem. J.* **22**, 1327.
Pohl & Rawicz (1919). *Hoppe-Seyl. Z.* **104**, 95.
Pugh & Raper (1927). *Biochem. J.* **21**, 1370.
Raper (1928). *Physiol. Rev.* **8**, 245.
—— (1932). *Biochem. J.* **26**, 2000.
Richter (1934). *Biochem. J.* **28**, 901.
Rosenheim & Starling (1933). *Chem. Ind.* **52**, 1056.
Rossbach (1884). *Berl. klin. Wschr.* **21**, 729.
Smith, Smith & Pincus (1938). *Amer. J. Physiol.* **121**, 98.
Stroud (1939). *Nature, Lond.*, **144**, 245.
Sutter (1936). *Ergebn. Enzymforsch.* **5**, 273.
Szent-Györgyi (1925). *Biochem. Z.* **162**, 399.
Thayer, Jordan & Doisy (1928). *J. biol. Chem.* **79**, 53.
—— Levin & Doisy (1931). *J. biol. Chem.* **91**, 791.
Wagreich & Nelson (1936). *J. biol. Chem.* **115**, 459.
Zeile (1931). *Hoppe-Seyl. Z.* **195**, 39.
Zondek (1934). *Skand. Arch. Physiol.* **70**, 133.

10. THE INEFFECTIVENESS OF OESTROGENS IN PREVENTING POLYNEURITIS

By W. W. WESTERFELD[1]

From the Department of Biochemistry, Oxford

(*Received 21 November 1939*)

CERTAIN phenanthrene derivatives have been reported [Rodriguez & Sarda, 1937] to be effective in delaying the onset of polyneuritis and in prolonging the lives of pigeons placed on a vitamin B_1-free diet. The substances reported to have this action were the male and female sex hormones (hombreol and progynon B) and vitamin D (vigantol), while the closely related corpus luteum hormone (proluton) was without effect. We have repeated the study with the female sex hormones, but have been unable to confirm the above observations. In our experience the administration of oestrogenic hormones has no effect whatsoever on the development of vitamin B_1 deficiency in pigeons.

EXPERIMENTAL

Two groups of 40 pigeons were each placed on a diet of polished rice for 30 days. One group was injected intramuscularly during this period with various esters of oestrone (acetate and benzoate) and oestradiol (dipropionate[2]); the other group served as a control. The results are shown in Table 1.

Table 1. *Lack of effect of oestrogens on the development of polyneuritis in pigeons on a vitamin B_1-free diet*

	Pigeons injected with oestrogens	Control pigeons
Total no. of pigeons studied	40	40
No. unaffected (except wt. loss) within 31 days	9	10
No. developing head retraction	20	18
Day (av.) of appearance of H.R.	19·3	21·4
Wt. loss (av.) at time of H.R.	31·3%	36·9%
No. dying without symptoms or before treatment	11	12
Day (av.) of death	24·6	27·2

The table shows a striking similarity between the control birds and those injected with oestrogens; there is no support for the belief that oestrogens protect the pigeons from vitamin B_1 deficiency. It is apparent that only 50% of the birds in either group developed the typical head retraction symptoms; this is more or less the usual experience in the routine preparation of avitaminous pigeons in this laboratory, and emphasizes the need for a large group of animals in such an experiment as this to prevent erroneous conclusions.

The pigeons that developed head retraction were placed in a warm room and given glucose orally; if the symptoms failed to improve, a standard preparation of vitamin B_1 was administered via the crop. The first dose of vitamin B_1 was effective in allaying the head retraction symptoms for an average of 9·2 days in the birds injected with oestrogens and 8·0 days in the controls.

[1] Fellow in the Medical Sciences of the National Research Council.

[2] The oestradiol dipropionate was generously supplied by Ciba, Ltd.

Subsequent doses of vitamin B_1 were effective for 5·0 days in the injected birds and 5·3 days in the controls.

Tissue respiration studies were carried out with minced pigeon cerebrum in Warburg manometers by the technique generally used in this laboratory [Passmore *et al.* 1933]. Na lactate was added as the substrate since it was also an indirect measure of pyruvate oxidation [Peters & Thompson, 1934]. For comparative purposes the brains of normal pigeons, pigeons that showed a marked vitamin B_1 deficiency, and avitaminous pigeons that had received a prolonged treatment with oestrogens were included, and in each case the effect of adding 1 μg. of oestradiol to the respiring tissue was simultaneously determined.

Table 2. *O_2 consumption of pigeon cerebrums from normal, B_1-deficient, and oestrogen-injected B_1-deficient pigeons*

Tissue alone and also in the presence of 1 μg. oestradiol *in vitro*. Measurements at 15 or 30 min. intervals in μl. O_2 per g. wet tissue per hr.

	Normals		B_1-deficient		B_1-deficient oestrogen-injected	
Time min.	Control	+ Oestradiol *in vitro*	Control	+ Oestradiol *in vitro*	Control	+ Oestradiol *in vitro*
15	2135	2250	2000	2105	2115	2140
30	1980	2065	1815	1920	1940	1970
45	1860	1965	1670	1775	1750	1790
60	1770	1880	1545	1640	1620	1650
90	1615	1755	1310	1400	1405	1430
120	1470	1565	1145	1220	1240	1260

The results are shown in Table 2. In all cases the oestrogen appeared to have a slight stimulating effect, but the differences were statistically insignificant. No significant difference could be detected in the O_2 utilization of the cerebrums of avitaminous pigeons whether or not they had been previously injected with oestrogens. Thus oestrogen *in vivo* failed to prevent the significant decrease in respiration due to vitamin B_1 deficiency. There was likewise no significant increase in the respiration of avitaminous cerebrums upon the addition of oestradiol *in vitro*, such as is given by vitamin B_1 in the catatorulin test.

Summary

The administration of oestrogenic hormones was without effect upon the development of polyneuritis in pigeons on a vitamin B_1-free diet.

There was no significant increase in the respiration of avitaminous cerebrums when oestrogen was added *in vivo* or *in vitro*.

The author wishes to express his thanks to Prof. R. A. Peters for suggesting this research and for the necessary aid in carrying it out.

REFERENCES

Passmore, Peters & Sinclair (1933). *Biochem. J.* **27**, 842.
Peters & Thompson (1934). *Biochem. J.* **28**, 916.
Rodriguez & Sarda (1937). *Z. Vitaminforsch.* **6**, 193.

II. THE PHOTOCHEMICAL DECOMPOSITION OF *l*-ASCORBIC ACID. II

By CHARLES LAURENCE ARCUS
and SYLVESTER SOLOMON ZILVA[1]

From the Division of Nutrition, Lister Institute, London

(*Received 28 November 1939*)

In a recent investigation [Kellie & Zilva, 1938] it was shown that in the presence of air, *l*-ascorbic acid in aqueous solution, when exposed to ultraviolet light, was oxidized without the aid of sensitizers to dehydroascorbic acid. Furthermore, it was found that even in the absence of atmospheric oxygen the exposure to this light brought about a destruction of the vitamin. In the anaerobic experiments in which small quantities of ascorbic acid were used the amount of dehydroascorbic acid present after the exposure at *p*H 7 was so small as to throw doubt on its formation. At *p*H 3 on the other hand although the quantity of ascorbic acid which was decomposed by the ultraviolet light was less than at *p*H 7, the reduction of the residual solution with H_2S produced an appreciable rise in the indophenol-reducing capacity of the solution. That this actinic decomposition of *l*-ascorbic acid under anaerobic conditions was not due to oxidation brought about by the last traces of O_2 which could not be removed by repeated exhaustion and refilling with N_2 was shown by the fact that the quantities of ascorbic acid which disappeared after prolonged exposure required considerably larger amounts of O_2 for their oxidation than those which could possibly be present in solution even without previously removing from it the dissolved air.

This observation on the anaerobic photolysis of *l*-ascorbic acid required further elucidation and experiments for this purpose are described below. In the original investigation ascorbic acid was determined by titration with 2:6-dichlorophenolindophenol and any rise in the indophenol-reducing capacity after reduction with H_2S was assumed to be dehydroascorbic acid. The determination of pure *l*-ascorbic acid in aqueous solution by titration with indophenol is beyond criticism and great reliance can also be placed on the interpretation that the rise in the indophenol-reducing capacity in the *l*-ascorbic acid solutions exposed to the light in the presence of air after treatment with H_2S is due to the reduction of the reversibly oxidized form of the compound produced by the oxidation of some of the ascorbic acid. In the case of the anaerobic experiments, however, it was considered desirable to obtain additional evidence for the assumption that the increase in the reducing capacity of the exposed solutions after treatment with H_2S was really due to the formation of *l*-ascorbic acid. A method for the determination of ascorbic acid based on the production of furfuraldehyde was therefore employed in the present investigation. The results obtained with this method were similar to those observed when indophenol was used, thus supporting the view that even under anaerobic conditions oxidation of ascorbic acid with the formation of dehydroascorbic acid takes place during the exposure of the reduced compound to ultraviolet light.

[1] Member of the Scientific Staff, Medical Research Council.

Determination of l*-ascorbic acid as furfuraldehyde*

The procedure adopted by us was based on the observation of Herbert *et al.* [1933] that by heating *l*-ascorbic acid with boiling 12% HCl it readily gave furfuraldehyde which could be determined as the phloroglucide yielding 87% of the theoretical value. As the application of the customary formula for the correction of the solubility of the phloroglucide yielded fictitious results with the quantities of ascorbic acid with which we worked we had recourse to the following empirical method which was found to be sufficiently accurate for our purpose. To a solution calculated to contain about 30 mg. of ascorbic acid were added half the volume of conc. HCl and sufficient 12% HCl to bring the volume up to 100 ml. This solution was then distilled over a free flame at first slowly and then at a rate yielding 30 ml. of distillate per 10 min. until 360 ml. of distillate were collected. The volume in the distilling flask was kept constant by periodical additions of 50 ml. 12% HCl. A quantity of phloroglucinol (about $2\frac{1}{2}$ times the weight of the expected furfuraldehyde) dissolved in 12% HCl was added to the filtered distillate with stirring. The solution was made up to 400 ml. and allowed to remain at room temperature overnight to allow the black precipitate to separate completely. The precipitate was then collected by filtration in a sintered-glass crucible, washed with 150 ml. of water without being allowed to become dry, dried at 100° for 4 hr. and weighed after cooling in a desiccator. The equivalent quantity of *l*-ascorbic acid was ascertained from a graph (Fig. 1)

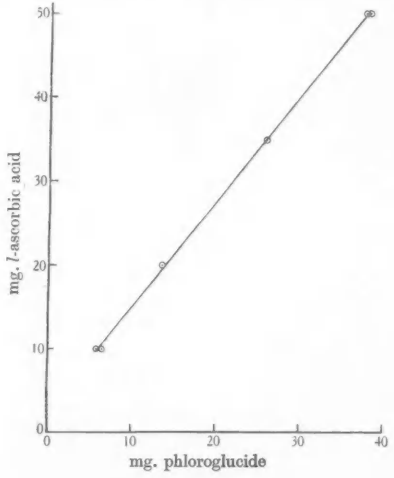


Fig. 1.

which was constructed by plotting the weights of the phloroglucide against the weights of *l*-ascorbic acid from which they were obtained. We have ascertained that solutions of dehydroascorbic acid distilled under the above conditions yielded a small quantity of phloroglucide which corresponded approximately to 10% of its equivalent of ascorbic acid. As will be seen later the quantities of dehydroascorbic acid involved in our experiments were of an order that could

not seriously vitiate the assessment of the *l*-ascorbic acid in the exposed solutions before they were reduced with H_2S. Roe [1936] claims that dehydroascorbic acid does not yield any furfuraldehyde at all when boiled with HCl.

The formation of dehydroascorbic acid from l-*ascorbic acid by irradiation under anaerobic conditions*

As the rise in the indophenol-reducing capacity of irradiated *l*-ascorbic acid solutions after treatment with H_2S was more marked in acid than neutral solution these experiments were carried out at *p*H 3·6. 50 mg. of *l*-ascorbic acid were dissolved in 100 ml. of sodium acetate-acetic acid buffer solution prepared with quartz-distilled water which was boiled and cooled immediately before use, and the solution was irradiated in a quartz tube of 125 ml. capacity. Before irradiation the tube was evacuated 3 times and filled with pure N_2 3 times and the solutions were therefore finally irradiated in N_2 at atmospheric pressure. The quartz tube was exposed for 4 hr. to a Hewittic Y 2 mercury lamp at a distance of 9 in. with constant turning. The temperature of the solution gradually rose to 46° during this time. After cooling 1 ml. 1 % NaCN was added to the irradiated solution and 40 ml. were removed for furfuraldehyde determination. At the same time 5 ml. were titrated with indophenol. A further 45 ml. were then placed in a wash bulb through which H_2S was passed for 5 min. and, after sealing the vessel, were allowed to remain overnight. After thoroughly displacing the H_2S by a current of pure N_2 the following day, the furfuraldehyde content was determined in 40 ml. and another 5 ml. were again titrated with indophenol.

Table 1. *Oxidation of* l-*ascorbic acid during anaerobic irradiation (initial concentration of* l-*ascorbic acid, 50 mg. per 100 ml.)*

	Indophenol-reducing capacity (mg. *l*-ascorbic acid per 100 ml.)				Determination as furfuraldehyde (mg. *l*-ascorbic acid per 100 ml.)			
Exp.	After irradiation	After reduction	Difference	Destroyed during irradiation	After irradiation	After reduction	Difference	Destroyed during irradiation
1	30·4	36·3	5·9	19·6	29·8	32·6	2·8	20·2
2	32·4	37·6	5·2	17·6	31·2	36·9	5·7	18·8
3	32·25	36·8	4·55	17·75	32·0	33·9	1·9	18·0
4	30·8	34·4	3·6	19·2	30·8	33·8	3·0	19·2
5	30·3	35·5	5·2	19·7	31·0	33·5	2·5	19·0
6	31·2	36·7	5·5	18·8	30·6	35·5	4·9	19·4

Table 2. *mg. of* l-*ascorbic acid per 100 ml. determined by indophenol titration*

Initial concentration	Time of exposure hr.	After irradiation	After reduction	Difference	Destruction per hr.
50	4·0	33·2	39·8	6·6	4·2
50	6·5	24·0	30·5	6·5	4·0
50	6·5	22·8	29·2	6·4	4·2
50	8·0	18·0	27·4	9·4	4·0
100	4·0	84·1	88·2	4·1	4·0
100	6·5	74·0	84·0	10·0	4·0

It will be seen from Table 1 that although both methods yielded consonant results for the total quantity of *l*-ascorbic acid destroyed during irradiation, this was not the case when the *l*-ascorbic acid in the exposed solution was determined

by these methods after reduction with H_2S. The results obtained by the furfuraldehyde method show nevertheless that considerable quantities of dehydroascorbic acid were formed during irradiation. This point will receive comment later. It may be mentioned here that the disappearance of the ascorbic acid was due to the action of the light and not to the rise in temperature of the exposed solution since there was no significant loss in similar solutions kept at 47° for 4 hr. in the dark.

The probable dependence of the disappearance of ascorbic acid on the intensity of the irradiation

In Table 2 are given figures for the disappearance of *l*-ascorbic acid in solution in two concentrations exposed during different periods of time. These experiments were carried out successively so that the actinic output was in all probability of the same intensity in each case. The fact which emerges from these results is that the quantity of *l*-ascorbic acid which disappeared per hour was the same in every experiment; the destruction was therefore proportional to the time of exposure. These results also indicate that the output of actinic energy by the lamp which we used was too low for the disappearance of *l*-ascorbic acid to be affected by variation in the concentrations employed. It would thus appear, as one would expect, that the chemical change is dependent on the intensity of the irradiation.

The protection of l*-ascorbic acid by glutathione during anaerobic irradiation*

Experiments were then carried out with the purpose of ascertaining whether the formation of dehydroascorbic acid takes place in solutions in which glutathione is present. A protective action of glutathione could be explained

Table 3. *Protection of* l*-ascorbic acid by glutathione during anaerobic irradiation*

Solution	Time of irradiation hr.	mg. *l*-ascorbic acid per 100 ml. Initial content	After irradiation	After reduction
With glutathione	0·5	47·5	47·8	46·5
Without glutathione	0·5	44·0	39·3	41·0
With glutathione	2·0	49·0	45·3	44·3
Without glutathione	2·0	45·0	35·0	34·8
With glutathione	4·5	48·8	40·1	38·8
Without glutathione	4·5	45·6	24·2	23·9
Control solution containing no ascorbic acid	4·5	0·5	2·5	1·0
With glutathione	Kept in dark in incubator at 47° for 4·5 hr.	52·5	53·0	52·3
Without glutathione		48·5	45·3	43·5

Table 4. *Screening of* l*-ascorbic acid during anaerobic irradiation*

Composition of screening solution	mg. *l*-ascorbic acid per 100 ml. Initial content	After irradiation	After reduction
Water only	48·0	17·0	16·0
0·100 g. glutathione in 50 ml. recently boiled and cooled quartz distilled water	46·5	40·8	39·0
0·34 g. KH_2PO_4, 2·0 ml. *N* NaOH in 50 ml. of solution, *p*H 7·4	47·3	17·0	17·5
0·34 g. KH_2PO_4, 2·3 ml. *N* NaOH, 0·100 g. glutathione, in 50 ml. of solution, *p*H 7·4	47·3	42·5	41·5

either by its reduction of the formed dehydroascorbic acid or by its absorption of rays responsible for the photochemical oxidation of the *l*-ascorbic acid. Two experiments were performed with this end in view. In both experiments the solutions were exposed at the physiological *p*H of 7·4.

In the first experiment 50 mg. of *l*-ascorbic acid, 0·68 g. KH_2PO_4 and 4·23 ml. of *N*/1 NaOH were made up to 100 ml. with quartz-distilled water (recently boiled and cooled). To 50 ml. of this solution 0·1 g. of crystalline glutathione and 0·33 ml. of *N*/1 NaOH were added. Both solutions were then at *p*H 7·4 and were placed in quartz flasks which were evacuated, filled with nitrogen and irradiated as before. Aliquot portions of solutions were titrated with indophenol at *p*H 3 before and immediately after each exposure. The exposed solutions were also reduced with H_2S and titrated again. It will be seen from Table 3 that in the presence and in the absence of glutathione there was a progressive disappearance of *l*-ascorbic acid during the exposure but, whilst in presence of glutathione after 4½ hr. irradiation the loss was of the order of 20%, in its absence the destruction amounted to about 45%. This observation leaves no doubt that the glutathione had a protective action on the actinic decomposition of the *l*-ascorbic acid. It may be noted that no dehydroascorbic acid could be demonstrated in these solutions since there was no increase in the indophenol titre after reduction with H_2S.

Fig. 2. *A*, layer of liquid paraffin employed to prevent oxidation when glutathione was present in the screening solution; *B*, atmosphere of nitrogen; *C*, screening solution; *D*, *l*-ascorbic acid solution.

In order to show that the protective action of the glutathione was mostly, if not entirely, due to its absorptive capacity for the active radiation, the following experiment was devised. 10 ml. of a solution of *l*-ascorbic acid, prepared as in the preceding experiment and also buffered at *p*H 7·4, were placed in a small quartz tube (approximate capacity 15 ml.). This tube was evacuated and filled with N_2 as before and placed concentrically within a larger quartz tube as shown in Fig. 2. The *l*-ascorbic acid was then exposed for 4½ hr. when the screening solution consisted of water, buffer or solutions containing 0·2% of glutathione. It is seen from Table 4 that the glutathione had in this case a protective action of the same order as in the preceding experiment. Also in this experiment, owing to the high *p*H, no dehydroascorbic acid was found.

Discussion of results

The results show conclusively that about two-thirds of the increase in the indophenol titration values after reduction of the exposed solutions of *l*-ascorbic acid could be traced to the formation of dehydroascorbic acid. The remaining third may have been due either (*a*) to the formation of some unknown substance during irradiation which, like dehydroascorbic acid, on reduction with H_2S yielded an indophenol-reducing substance, or (*b*) to some chemical change incurred during the reduction of the solution which interfered with the furfuraldehyde determination. Whatever be the explanation of the lack of agreement

in the ascorbic acid contents of the irradiated and reduced solutions as determined by the two methods, it is noteworthy that dehydroascorbic acid was formed beyond doubt during the exposure to ultraviolet light in quantities requiring more O_2 than the traces which could possibly have been present in the *l*-ascorbic acid solutions after repeated exhaustion and refilling of the tube with N_2.

Since Tian [1911] claimed that traces of O_2 together with H_2O_2 are formed when water is exposed to ultraviolet light of 190 mμ, attempts were made by us to detect the formation of O_2 and of H_2O_2 during irradiation of buffer solutions under our experimental conditions, in order to ascertain whether the oxidation was due to this agency. Utilizing the $Ti(SO_4)_2$ test for H_2O_2 and the $Mn(OH)_2$ test for O_2 we could not detect the presence of either. That O_2 and H_2O_2 formed from water during the irradiation could not be responsible for the production of dehydroascorbic acid was also shown by the fact that the reversible oxidation of *l*-ascorbic acid took place even when it was screened during exposure by water which according to Tian's claims should have filtered out the short wave ultraviolet light capable of decomposing water. One is, therefore, forced to the conclusion that the ultraviolet light decomposed some of the ascorbic acid in solution with the formation of an oxidizing substance or substances which in their turn dehydrogenated some of the undecomposed *l*-ascorbic acid. The additional observation that in the presence of glutathione *l*-ascorbic acid in solution is protected from this process of dehydrogenation suggests that such oxidation is not likely to take place *in vivo* even if ultraviolet light were to penetrate superficial tissues containing vitamin C. Furthermore, any dehydroascorbic acid formed would simultaneously be reduced by the glutathione present.

Summary

The observation made by Kellie & Zilva that dehydroascorbic acid is formed by exposing *l*-ascorbic acid anaerobically in buffer solution to ultraviolet light has been confirmed by using the furfuraldehyde method for the determination of ascorbic acid. The evidence shows that the extent of the reaction is dependent on the intensity of the ultraviolet radiation.

It is assumed that some ascorbic acid is decomposed during the exposure with the formation of a substance or substances capable of dehydrogenating some of the undecomposed *l*-ascorbic acid.

Glutathione, whether present in solution with the *l*-ascorbic acid or acting as a screen in a buffer solution, exercises a protective action against dehydrogenation.

It is improbable that any ultraviolet light penetrating superficial tissues containing *l*-ascorbic acid would bring about the oxidation of the vitamin *in vivo*.

One of us (C. L. A.) is indebted to the Medical Research Council for a whole time grant. We should also like to express our thanks to Messrs Roche Products Ltd. for a gift of *l*-ascorbic acid.

REFERENCES

Herbert, Hirst, Perceval, Reynolds & Smith (1933). *J. chem. Soc.* p. 1270.
Kellie & Zilva (1938). *Biochem. J.* **32**, 1561.
Roe (1936). *J. biol. Chem.* **116**, 609.
Tian (1911). *C.R. Acad. Sci., Paris*, **152**, 1012.

12. THE COLOURING MATTERS OF *PENICILLIUM CARMINO-VIOLACEUM* BIOURGE, WITH A NOTE ON THE PRODUCTION OF ERGOSTEROL BY THIS MOULD

By H. G. HIND

University College, Nottingham, and the Lister Institute, London

(*Received 27 November 1939*)

In recent years great advances have been made in the chemistry of moulds, and many compounds new to organic chemistry have been isolated. Much attention has been devoted to colouring matters; many of these have been obtained chemically pure and in some instances the constitutions of the compounds have been determined, particularly by H. Raistrick and co-workers.

Cultures of *P. carmino-violaceum* Biourge grown on carbohydrate media are characterized by a colouring of the medium, yellow when acid and claret red when alkaline. This pigment was observed by Krause & Ellis [1937] who rightly assumed that the colour changes in the medium with change of *p*H were due to indicator properties of the pigment. It was further pointed out that the pigment was precipitated by acid from alkaline solution; however, it was not isolated in a state of chemical purity. The mycological characteristics of *P. carmino-violaceum* are described by Thom [1930].

The present investigation has revealed that *P. carmino-violaceum* Biourge produces two pigments of molecular formulae $C_{20}H_{16}O_7$ and $C_{16}H_{12}O_6$ respectively. These compounds are indistinguishable by their colour reactions, both probably being complex hydroxyanthraquinones. The name carviolin is suggested for the compound $C_{16}H_{12}O_6$ and carviolacin for the compound $C_{20}H_{16}O_7$.

Carviolacin is a monomethyl ether, and, on account of its high carbon content, must possess a cyclic structure. Reduction with zinc dust of an alkaline solution of the pigment caused the colour to change from red to orange, and addition of H_2O_2 or shaking with air reversed the colour change; alizarin shows similar reactions with the above reagents, but in this case the colour change is purple to orange. Although the structure of carviolacin has not yet been determined, the following points have been established:

(1) Treatment with dimethyl sulphate and alkali gives a compound $C_{23}H_{22}O_7$ which is a trimethoxy derivative of the pigment.

(2) Zinc dust distillation yields 2-methylanthracene.

Carviolin is also a monomethyl ether. Its general reactions indicate quinonoid properties. The tendency of the compound and its derivatives to retain solvent of crystallization has rendered investigation difficult. After many analyses and rigorous purification the formula $C_{16}H_{12}O_6$ was assigned to the compound.

Its structure has not been determined but certain of its reactions have been studied.

(1) Acetylation with acetic anhydride and pyridine yields a triacetate having invariably one molecule of water of crystallization.

(2) Methylation with dimethyl sulphate and alkali gives a compound $C_{19}H_{18}O_6$ which is a trimethoxy derivative of $C_{16}H_{12}O_6$.

(3) On simultaneous reduction and acetylation a penta-acetate of the leuco-compound was obtained which had a very strong blue fluorescence in acetic acid solution.

Experimental

Cultures

The mould culture used in the investigation was isolated and kindly presented by Miss M. Ellis of University College, Nottingham. A culture of the mould was identified as *P. carmino-violaceum* Biourge by Prof. Thom of the U.S. Department of Agriculture.

A culture of *P. carmino-violaceum* Dierckx, supplied by the Centraalbureau voor Schimmelcultures, Baarn, did not produce any anthraquinone-like pigments under the same experimental conditions as those used in the following experiments.

Cultural conditions

Since the pigments were more readily obtainable from the mycelium than from the metabolic solutions, it was desirable that the pigments should be retained in the mycelium as long as possible. Early experiments had demonstrated that the pigments are soluble in alkali and therefore the medium had to remain acid during the period of growth. A study of the effect of the source of carbon on the rate of change of *p*H during growth was made. The mineral salts used were the same throughout but different sugars were employed in the various tests. The cultures were set up in 300 ml. conical flasks and each flask contained 100 ml. of solution of the following composition: carbohydrate 5 g.; KNO_3 1 g.; KH_2PO_4 0·5 g.; $MgSO_4, 7H_2O$ 0·25 g.; H_2O 100 ml. The cultures were grown at room temperature. The *p*H was determined from time to time by means of a B.D.H. capillator. In most cases the experiments were carried out in duplicate. The compounds tested as source of carbon were maltose, fructose, glucose, sucrose, lactose and glycerol.

The maltose medium apparently gave a good yield of the pigment but it was unsuitable because the *p*H rose too quickly, with the result that the pigment diffused into the medium. In the lactose medium the mould grew somewhat less vigorously and pigment production was apparently much retarded. Media containing carbohydrates other than lactose became yellow after a few days, while in the case of the lactose medium no such coloration was observed and it was only after about 20 days in this medium that an orange pink tinge appeared. For the purpose of pigment production and maintenance of acidity which facilitated isolation the glycerol medium was the most suitable and it was therefore employed.

500 ml. quantities of the culture medium were placed in 2·5 l. culture bottles which were sterilized in steam for 1·5 hr. on each of three successive days, and then kept for a further 7 days to ensure that sterilization was complete. They were then inoculated with spore suspensions in sterile water, the spores being obtained from stock cultures on solid media. The bottles were kept at room temperature.

Extraction of the pigments

The mycelium was removed from the culture bottles when the *p*H had risen to about 6·5 (usually 10–12 weeks). The mycelium was then undulating and white to pale bluish-green with reddish droplets on the surface, exuded from the mycelium. The culture medium was orange-yellow in colour, and the reverse of the mat was deeply coloured a rich golden brown. The mycelium was filtered

from the medium, washed with water and further treated by one of the following methods.

Method 1. The washed mycelium was pressed dry and extracted in a Soxhlet extractor with acetone. The residue from the acetone extraction was a greyish-white material containing no colouring matter. The acetone and water were removed *in vacuo* from the extract and the resulting dark brown pasty mass was extracted twice with a large amount of light petroleum (B.P. 40–60°). The residue was then extracted with hot absolute alcohol, 100 ml. per g. of solid, and filtered. The extract contained the anthraquinone-like pigments, and left undissolved some dark brown material which constituted about half the weight of the petroleum-insoluble fraction. To the alcoholic solution, 2 vol. of light petroleum (B.P. 40–60°) were added and a further quantity of dark brown material was precipitated. This was discarded. On removal of the solvents a brown solid remained which was still greasy to the touch and contained the anthraquinone-like pigments. This constituted about 3% of the dry weight of the mycelium. Further purification was effected by extraction with light petroleum.

Method 2. The mycelium was dried over $CaCl_2$ in a desiccator, and the dried material was powdered by grinding in a small hand mill. The powdered mycelium was then extracted with light petroleum (B.P. 40–60°) in a Soxhlet extractor. This removed the greater part of the fat and facilitated later work. The pigment from the mycelium was then extracted continuously for 15 hr. with acetone. The acetone was removed and the acetone residue in turn was continuously extracted with ether for 15 hr. when the anthraquinone-like pigments passed into the ether. This method was less satisfactory than method 1 as the mycelium darkened during drying and less effective extraction of the pigment was obtained.

The preparation properties and derivatives of carviolin

Isolation as acetate. Raistrick *et al.* [1934] described the purification of anthraquinones from the extracts of the mycelia of species of *Helminthosporium* by acetylating the crude material and fractionating the acetates. The same method has been particularly applicable for separating the pigments of *P. carmino-violaceum* Biourge.

The pigment concentrates obtained as described above were acetylated for 4 hr. on the water bath at 100° with acetic anhydride (10 ml. per g.) and pyridine (2 ml. per g.). The cooled reaction mixture was poured into 20 vol. of cold water and filtered. On crystallizing from acetic acid, a compound separated which crystallized in pale cream-green needles, M.P. (after four recrystallizations from acetic acid) 209°. It was insoluble in cold dilute NaOH, but readily dissolved on warming to form a blood-red solution. It was soluble in conc. H_2SO_4 to give a red solution. (Found (on specimens from different batches of pigment, dried at 110° *in vacuo*): C, 59·4, 59·7; H, 4·7, 4·5; MeO, 7·5, 7·2, 7·0; acetyl, 28·7%. $C_{16}H_9O_3(O.OC.CH_3)_3$ H_2O requires C, 59·5; H, 4·5; MeO, 7·0; acetyl, 28·3%.)

The recrystallized acetate was hydrolysed by refluxing with $2N$ H_2SO_4 containing 40% alcohol for 4 hr., during which time the acetate slowly dissolved, giving finally a clear deep yellow solution, from which crystals of carviolin separated on cooling. The pigment was recrystallized 3 times from alcohol, once from pyridine and finally 3 times from acetic acid, from which solvent it separated as chrome yellow needles, M.P. 286°. (Found (for compound dried at 160° *in vacuo*): C, 63·7; H, 4·2; MeO, 10·7%. $C_{16}H_{12}O_6$ requires C, 64·0; H, 4·0; MeO, 10·3%.) The final crystallizations were performed to remove

alcohol of crystallization, which was retained most tenaciously. If the final crystallization was from alcohol, the methoxyl content as shown by Zeisel estimation was always high, even after vigorous drying.

Direct isolation of carviolin. The acetone extract (method 1), if filtered, invariably yielded a quantity of dark brown material which on drying turned yellowish in colour. 1 g. of this was extracted with dilute ammonium hydroxide which gave a blood-red solution and a dark insoluble residue. The solution was filtered and acidified with HCl, and a chrome yellow precipitate formed. This was suspended in 100 ml. water, heated to 80° and treated with alcohol until the solution was clear. On cooling, a chrome yellow crystalline pigment separated (0·1 g.). This was recrystallized 4 times from 50% alcohol and water and had M.P. 286°. (Found (on material dried at 110° *in vacuo*): C, 63·6; H, 4·3; MeO, 11·1%. $C_{15}H_9O_5(OMe)$ requires C, 64·0; H, 4·0; MeO, 10·3%.)

Carviolin is insoluble in water and HCl. It dissolves in dilute NH_4OH to give a clear blood-red solution, while in NaOH or KOH a bluer shade is obtained. Carviolin gives a red colour with conc. H_2SO_4 which changes to a reddish-purple on addition of boric acid.

The compound has acidic properties as shown by its solubility in alkali; carviolin cannot be extracted from NaOH by ether, but becomes extractable after acidification.

Carviolin dissolves in cold Na_2CO_3 to give a red solution, but is insoluble in bicarbonate. It is not a mordant dye. The pigment does not sublime in high vacuum at 200°.

Carviolin triacetate $C_{15}H_6O_2(O.CH_3)(O.COCH_3)_3$, H_2O. 40 mg. of carviolin were heated with 0·4 ml. of pyridine and 3 ml. of acetic anhydride for 3 hr. at 100°. The reaction mixture was cooled and 4 ml. of water were added when the product separated as minute cream-green needles (yield theoretical). These were recrystallized 3 times from acetic acid. M.P. 210° alone or mixed with a specimen isolated from the acetylated crude extract.

Carviolin trimethyl ether $C_{15}H_6O_4(OCH_3)_4$. 0·15 g. of carviolin was dissolved in 5 ml. acetone and 3 ml. dimethyl sulphate. Sufficient 2*N* NaOH was added from time to time to keep the reaction mixture alkaline. During the reaction carviolin trimethyl ether separated out crystalline. The acetone was evaporated from the alkaline solution and the product filtered (yield 0·12 g.). This was recrystallized from 96% alcohol in gold-coloured rods, M.P. 186°. (Found (on material dried at 110° *in vacuo*): C, 67·0; H, 5·15; MeO, 34·2%; mol. wt. (Rast), 348. $C_{15}H_6O_2(OCH_3)_4$ requires C, 66·7; H, 5·2; MeO, 36·2%; mol. wt. 342.)

Carviolin tribenzoate $C_{15}H_6O_2(OCH_3)(O.COC_6H_5)_3$. 40 mg. of carviolin were heated with 2 ml. pyridine and 5 ml. benzoyl chloride for 3 hr. on the water bath (100°). The cooled reaction mixture was poured into excess of water and the oily drops which separated were collected with 10 ml. chloroform. 2*N* Na_2CO_3 was added and the two layers left in contact for 30 min. with occasional shaking in rotary manner. The chloroform layer was separated; to it were added 40 ml. alcohol and the chloroform was evaporated on the water bath till crystallization commenced. On cooling, the benzoate separated quantitatively. It was recrystallized from alcohol and chloroform in the same manner without loss, since it was apparently insoluble in cold alcohol (yield 80 mg.; theoretical yield for tribenzoate of $C_{16}H_{12}O_6$, 81·6 mg.), M.P. 240°. (Found (dried at 110° *in vacuo*): C, 72·0; H, 4·2; MeO, 5·3%. $C_{37}H_{24}O_9$ requires C, 72·5; H, 3·9; MeO, 5·1%.)

Leuco-carviolin penta-acetate. 0·1 g. carviolin was dissolved in 8 ml. of acetic anhydride; 0·5 ml. pyridine and 0·5 g. magnesium powder were added in small

quantities at intervals while the material was kept at 100°. The evolution of H_2 by this method was more copious and satisfactory than when zinc dust was used. The product was filtered, cooled and poured into cold water when an oil separated which hardened and crystallized on standing. After standing overnight it was crystallized from acetic acid in which solvent it had a blue fluorescence. The compound was finally recrystallized 3 times from 60% acetic acid. Cream coloured rods, M.P. 247°. (Found (on material dried at 110° *in vacuo*): C, 61·0; 60·8; H, 5·0, 4·9; MeO, 6·6%. $C_{15}H_6(OMe)(O.COCH_3)_5$ requires C, 60·9; H, 4·7; MeO, 6·1%.) A product (M.P. 261°) was obtained by the above method from anthraquinone for comparison with the penta-acetate of leuco-carviolin. This compound was evidently oxanthranol diacetate [Liebermann, 1888]. It had a blue fluorescence which strongly resembled that of leuco-carviolin penta-acetate.

Zinc dust distillation. A zinc dust distillation carried out using 100 mg. of carviolin yielded a small quantity of distillate, but no crystalline material was isolated.

Preparation, properties and derivatives of carviolacin

Isolation. The mother liquors, obtained by crystallizing the acetate of carviolin, in the isolation of that compound (*q.v.*) were invariably deep brown in colour. These were evaporated to dryness *in vacuo* and then treated with 2*N* NaOH on the water bath for 20 min. Filtration removed a quantity of dark material which was discarded, and on acidifying the wine-red solution a brown precipitate separated which crystallized from dilute alcohol and water in light brown needles. On heating they darkened at 220° and softened with decomposition at 243°. (Found (on material dried at 110° *in vacuo*): C, 65·3; H, 4·1; MeO, 8·4%; mol. wt. (Rast), 410. $C_{20}H_{16}O_7$ requires C, 65·2; H, 4·4; MeO, 8·4%; mol. wt. 368.) Carviolacin has also been obtained directly from the mycelium, but the yield was poor. There is no doubt that carviolin and carviolacin are unchanged by the acetylation and hydrolysis, and the acetylation method gives a satisfactory separation of the pigments.

Carviolacin is indistinguishable from carviolin by its colour reactions and the colour of its solutions, and is apparently closely related to that pigment. It has a tendency to dissolve in Na_2CO_3. It is insoluble in bicarbonate and it is not a mordant dye. It is more soluble in aqueous solvents than is carviolin, and unlike this compound it is soluble to some degree in water and dilute HCl. Efforts to sublime carviolacin have failed.

Carviolacin trimethyl ether $C_{19}H_{10}O_3(OCH_3)_4$. 0·1 g. of carviolacin was dissolved in 8 ml. of acetone and 7 ml. of dimethyl sulphate were added. 2*N* alkali was added from time to time to keep the reaction mixture alkaline, and finally the acetone was evaporated on the water bath when the derivative separated (0·08 g.). This was recrystallized from alcohol as yellow needles, M.P. 214–215°. (Found (on material dried at 110° *in vacuo*): C, 67·3; H, 5·1; MeO, 30·2, 30·4%. $C_{19}H_{10}O_3(OMe)_4$ requires C, 67·3; H, 5·4; 4MeO, 30·2%.)

Acetylation of carviolacin. An acetyl derivative was prepared to study its properties as an aid in the pigment separation. 20 mg. carviolacin, 1 ml. acetic anhydride and 1 drop of conc. H_2SO_4 were heated on the steam bath for 2 hr. The reaction mixture was cooled and poured into water, but partially dissolved. The solution was extracted with 20 ml. of $CHCl_3$ and the two layers were separated. 20 ml. alcohol were added to the $CHCl_3$ extract and the $CHCl_3$ removed on the water bath. On standing overnight the acetate separated in yellow needles. These were recrystallized 4 times from alcohol and water (M.P. 230°).

Zinc dust distillation. 250 mg. of carviolacin were mixed with 1·4 g. of zinc dust and a further 0·2 g. zinc dust was added to cover the mixture in the distillation apparatus. On heating, a colourless crystalline compound collected in the cooler parts of the tube, together with some yellow oily liquid. On cooling, the contents of the tube were extracted with ether and the extract was filtered. This gave a yellowish solution, which showed a green fluorescence. The ether was evaporated and the brown crystalline material was recrystallized twice from alcohol. It formed plates with greenish fluorescence (M.P. 203°) with a tendency to sublime above 185°, and had M.P. 204° when mixed with 2-methylanthracene (M.P. 206°). The mixed M.P. with anthracene (M.P. 212°) was 189°. There seems little doubt that the compound obtained by zinc dust distillation of carviolacin is 2-methylanthracene.

Ergosterol

In the present work it was found that on concentrating the petroleum extracts of the dried mycelium (method 2) colourless needles separated which crystallized readily from alcohol. These crystals were insoluble in dilute HCl and dilute NaOH, and had M.P. (after recrystallizing 4 times) 156°. The following positive reactions indicate that the substance was a sterol.

(1) Conc. H_2SO_4 added to an acetic anhydride solution gave a red colour which changed rapidly to green.

(2) A blue colour was given with $CHCl_3$ and $CCl_3.COOH$ [Rosenheim, 1929].

70 mg. of material were heated in a solution of 2 ml. benzoyl chloride and 0·5 ml. pyridine for 5 min. and the cooled solution was shaken with 25 ml. of *N* Na_2CO_3 for 5 min. The product was collected (yield theoretical) and recrystallized from alcohol 5 times. It had M.P. 164°, the M.P. of ergosterol benzoate from ergosterol isolated from *P. puberulum* Bainier [Birkinshaw *et al.* 1931] being 163°.

SUMMARY

Investigation of the colouring matters of *P. carmino-violaceum* Biourge has led to the isolation of two complex anthraquinones which have been named carviolin ($C_{16}H_{12}O_6$) and carviolacin ($C_{20}H_{16}O_7$) respectively. The preparation of various crystalline derivatives of these pigments are described and throw light on their constitutions. Ergosterol was isolated from the mould.

I am deeply indebted to Prof. J. M. Gulland and Prof. H. Raistrick for much helpful advice.

REFERENCES

Birkinshaw, Callow & Fischmann (1931). *Biochem. J.* **25**, 1977.
Krause & Ellis (1937). *Ann. Bot., Lond.*, N.S. **1**, 499.
Liebermann (1888). *Ber. dtsch. chem. Ges.* **21**, 1172.
Raistrick, Robinson & Todd (1934). *Biochem. J.* **28**, 559.
Rosenheim (1929). *Biochem. J.* **23**, 47.
Thom (1930). The Penicillia. London.

13. THE ESTIMATION OF BARBITURATES IN BLOOD

By G. A. LEVVY

From the Department of Pharmacology, University of Edinburgh

(*Received 25 November 1939*)

In connexion with a study of the distribution and fate in the body of the shorter-acting barbiturates, evipan and pentothal, it was necessary to carry out estimations of these drugs in blood and other biological media. The quantitative extraction of these unstable compounds presents some difficulty, and none of the methods given in the literature for dealing with more stable barbituric acids [Brundage & Gruber, 1937; Fischer, 1939; Herwick, 1933; Linegar *et al.* 1935; Koppanyi *et al.* 1934] was found suitable. It was thus necessary to work out a satisfactory procedure.

In clinical work the need has long been felt for a simple method of determining barbiturate concentrations in blood. The method described here, in addition to giving satisfactory extraction of evipan or pentothal, has many advantages over previously described methods in the rapid, routine determination of any type of barbituric acid in blood. It was found that 20 ml. blood from a patient on only a moderate dosage of barbitone were quite sufficient for examination. A study has been planned of the use of the procedure as a routine clinical test. The method should also be of value in toxicology.

For quantities of 1 mg. or more of barbiturate, the observed maximum error for the complete procedure is 20%, and for smaller quantities the error is correspondingly greater. This large experimental error is to be expected, since the colorimetric estimation after extraction is itself only accurate to within 10%, and since the drugs in question are unstable and difficult to obtain absolutely pure. The method is considered sufficiently accurate to meet the requirements of the pharmacological research for which it was designed, and it is felt that efforts to determine evipan or pentothal more accurately would defeat their own end, owing to the instability of these compounds. Within the limits given, the method is very reliable; three determinations may be carried out in 5 hr., and require no attention for much of that time.

Some experiments have been carried out on the extraction of barbiturates from tissues, other than blood, with promising results. The technique may be applicable to the extraction of drugs other than barbiturates.

Method

Evipan and pentothal are completely insoluble in acid and neutral solutions, and in alkaline solution they are extremely unstable; hence no method of extraction involving a protein precipitation can be used. Methods of direct liquid extraction from acid media with large volumes of a fat solvent can hardly be considered suitable for a routine quantitative estimation. The technique finally adopted was to dry the blood with anhydrous sodium sulphate so as to give a fine powder which could be extracted in a Soxhlet apparatus with a fat solvent. After purification, the barbiturate was then estimated by Koppanyi's colour reaction with cobalt acetate and *iso*propylamine in chloroform

[Dille & Koppanyi, 1934]. While this work was in progress, Griffon & Breton [1939] published a method for the detection of barbiturates, involving the use of sodium sulphate as a drying agent. These authors recommend as a qualitative test for barbituric acids a modification of Koppanyi's reaction, using diethylamine in alcohol in place of *iso*propylamine in chloroform; this has been examined and found to be inferior to the original for quantitative work.

To acidify the blood before drying, sodium dihydrogen phosphate was added. The use of this has the advantage that no neutralization of the extract is necessary, nor is there any release of pigments from the blood as with strong acids. It was considered desirable to carry out the extraction at as low a temperature as possible, and ether was therefore at first used as solvent, but the fats extracted from the dried blood were found to interfere in the colour reaction. This difficulty was overcome by using the mixture of equal parts of ether and light petroleum (B.P. 30–40°), which Brundage & Gruber [1937] recommend for the elution of barbiturates from charcoal. With this solvent a crystalline residue practically free from fat was obtained.

The barbiturate thus extracted from blood could, in most cases, be taken up straight away in chloroform and estimated, but occasionally it was contaminated by a small quantity of phospholipins; this contamination always occurred with extracts of tissues other than blood. The contaminants must be removed to obtain a true colour match with the standard, and, as a routine precaution, all ethereal extracts were shaken with a mixture of charcoal and magnesium oxide [Fischer, 1939] for a few minutes before evaporation. Great care must be exercised in the use of an adsorbing agent for the purification of

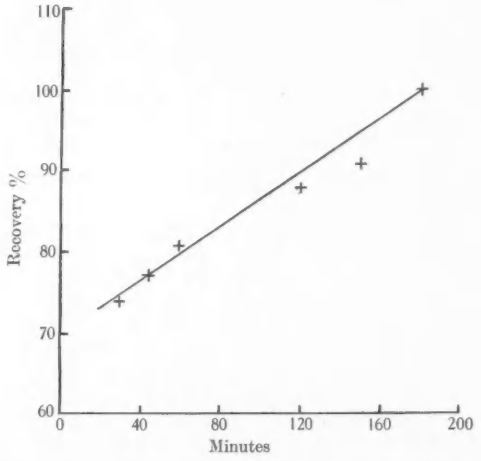


Fig. 1. Recoveries of barbitone from blood for different times of extraction.

a barbiturate solution. Even under the conditions specified below for purifying extracts there was found to be a very slight, but quite definite, adsorption of the drug from a barbiturate solution in ether and light petroleum. Unfortunately, no alternative scheme of purification was found practicable.

All ether used in the estimation must be freed from oxides, as the smallest traces of these prevent the reaction with cobalt acetate, and they are not removed in the treatment with charcoal and magnesium oxide. Estimations can best be carried out serially.

Fig. 1 shows the relation found between time of extraction and percentage recovery of 10 mg. sodium barbitone added in 1–2 ml. water to 20 ml. citrated blood. From this it is seen that extraction is complete, within the experimental error of the colorimetric estimation, in 2·5–3 hr. Barbitone being one of the least soluble of the barbiturates in the solvent used, all extractions were continued for this time.

Experimental

Reagents

Extraction mixture. Oxide-free ether is prepared weekly by washing with ferrous sulphate solution and water, and distilling into a bottle containing clean copper wire. It is mixed before use with an equal volume of light petroleum (B.P. 30–40°) in a measuring cylinder, the walls of which retain any water that may separate.

Charcoal and magnesium oxide mixture. The charcoal (Merck's "Ultracarbon") is washed with oxide-free ether containing a few drops of glacial acetic acid, and then with ether till free from acid. Three parts prepared charcoal are mixed with one part magnesium oxide.

Cobalt acetate solution. 1 % in absolute methyl alcohol.

iso*Propylamine solution.* 5 % by volume in absolute methyl alcohol.

Standard solutions for colorimeter. Solutions of the barbituric acid (not the sodium salt) under examination are prepared in chloroform in the following concentrations: 0·02, 0·04, 0·06, 0·08, 0·10, 0·12 %. These are stored in dark, glass-stoppered bottles.

Procedure

In a mortar of 250 ml. capacity, 20 ml. blood (coagulated or citrated) is mixed with 2 g. NaH_2PO_4. There are then added, in small portions with constant grinding, 40 g. anhydrous Na_2SO_4. After thorough mixing of blood and sulphate, the mortar is placed in a desiccator for 10–15 min. to allow complete drying to occur. The dry cake obtained is broken up and packed into a 100 ml. Soxhlet thimble. It is advisable to place a filter paper in the top of the filled thimble to prevent displacement of the powder. Extraction is carried out for 2·5–3 hr. at 50° with 80 ml. of ether-petroleum mixture. The addition of a few glass beads to the ether is necessary to facilitate even boiling.

At the end of the extraction the contents of the flask are shaken for a few minutes with about 0·25 g. charcoal and magnesium oxide. This is then removed by filtration directly into a distilling flask through a Jena sintered glass funnel, washing with small quantities of ether-petroleum, and the filtrate is distilled at 50°. The crystals of barbiturate which remain in the flask are taken up in a small quantity of chloroform and transferred to a 10 ml. graduated centrifuge tube, the solution being made up to a convenient volume. Owing to the difficulty of measuring chloroform, the loss of accuracy involved in the use of graduated tubes for this purpose was considered to be justified by the gain in rapidity of working.

To 2 ml. of the chloroform solution are added 0·1 ml. cobalt acetate solution and 0·6 ml. *iso*propylamine solution, and the colour is compared in a micro-

colorimeter with that given by 2 ml. of one of the standard solutions similarly treated. It may be necessary to dilute or concentrate the unknown solution to bring it within the range of concentrations covered by the standards.

Results

Figures are shown in Table 1 for the recoveries of four barbituric acids added, as their sodium salts in 1 ml. water, to 20 ml. citrated blood. Blank determinations were carried out, using double quantities of citrated or fresh coagulated blood to which no barbiturate had been added. After distilling off the ether-petroleum mixture, the flask was washed out either with 2 ml. chloroform or with one of the standard barbiturate solutions. No colour was produced with cobalt acetate and *iso*propylamine in the former case, and in the latter no change was observed in the intensity or shade of the colour given by the standard solution. From the latter series of observations it appears that, under the stated conditions, there is extracted from blood no substance which could cause a false result through an additive or potentiating effect on the reaction between barbiturates and cobalt acetate.

Table 1

Barbiturate	Added mg.	Found mg.	Recovery %
Barbitone	10·4	9·5	91
	8·9	8·9	100
	4·5	4·6	102
	1·04	1·05	101
	0·45	0·56	124
Phenobarbitone	10·3	8·5	83
	5·1	4·3	84
	0·51	0·41	80
Pentothal	9·3	7·8	84
	4·6	4·3	94
	0·46	0·59	128
Evipan	9·9	9·3	94
	8·7	7·2	83
	8·7	8·8	101

Samples of blood (supplied by Dr H. Tod) from three patients, to whom barbitone had been regularly administered for years, were examined by this method (Table 2). The dosage was such as had been found to give the same degree of depression in each case.

Table 2

Case	Daily dose mg./kg.	Barbitone found mg./20 ml.
Mrs G.	20·2	1·1
Miss D.	16·5	1·1
Miss S.	16·0	1·0

Summary

A simple procedure has been developed for the extraction of unstable barbiturates from blood. It has been found satisfactory for use in the routine determination of any type of barbiturate.

The author desires to express his gratitude to Prof. A. J. Clark for his continued interest and advice, to Dr P. Eggleton for many valuable suggestions, and to Imperial Chemical Industries Ltd. for defraying the expenses of this work.

REFERENCES

Brundage & Gruber (1937). *J. Pharmacol.* **59**, 379.
Dille & Koppanyi (1934). *J. Amer. Pharm. Ass.* **23**, 1079.
Fischer (1939). *Mikrochemie*, **26**, 255.
Griffon & Breton (1939). *C.R. Soc. Biol., Paris*, **130**, 337.
Herwick (1933). *Arch. int. Pharmacodyn.* **45**, 160.
Koppanyi, Dille, Murphy & Krop (1934). *J. Amer. Pharm. Ass.* **23**, 1074.
Linegar, Dille & Koppanyi (1935). *J. Amer Pharm. Ass.* **24**, 847.

14. PORPHYRINURIC ACTION OF 2-(*p*-AMINOBENZENESULPHONAMIDO)PYRIDINE (M AND B 693). COMPARISON WITH SULPHANILAMIDE

By CLAUDE RIMINGTON

From the National Institute for Medical Research, London, N.W. 3

(*Received 29 November 1939*)

Experiments previously reported [Rimington, 1939; Rimington & Hemmings, 1939] have shown that sulphanilamide and certain related nitro- and amino-compounds cause a marked increase in urinary porphyrin excretion when administered to rats for some days. A theory of pigment metabolism has been put forward to explain the mechanism of this toxic action [Rimington & Hemmings, 1939; Rimington, 1939].

The toxicity of the drug 2-(*p*-aminobenzenesulphanilamido)pyridine (M and B 693) has been examined by Wien [1938], who stated that it did not cause porphyrinuria in rats when administered at levels of 3 g./kg. and 6 g./kg. respectively for 15 days. It was claimed that M and B 693 offered a big advantage over sulphanilamide on account of lower toxicity. It may be here remarked that an examination of the figures published by Wien suggest that, in fact, a small and irregular increase of porphyrin was commencing. Sulphanilamide produced a much more striking result.

Since this early publication and following the introduction of the drug into clinical medicine, a number of reports have appeared which indicate that M and B 693 may cause more severe toxic symptoms than was at first suspected. Thus haematuria and haemoglobinuria have been repeatedly observed and not infrequently granulocytopenia. In addition, skin rashes, often showing an association with exposure to ultraviolet or sun light, have been reported [Hallam, 1939; Ker-Gray, 1939, etc.], similar to those which have been encountered during sulphanilamide therapy.

Detailed study of the rates of absorption and excretion of M and B 693 has been undertaken by Long & Feinstone [1938], Marshall *et al.* [1938], Baines & Wien [1939], Stokinger [1939], Powell & Chen [1939], and others.

Long & Feinstone stated that the drug is less readily and more irregularly absorbed by human beings than is sulphanilamide. Moreover, they consider that relatively more of the drug is conjugated in man than is the case when sulphanilamide is administered. An examination of their data hardly supports this conclusion. From the figures quoted, the mean value for the proportion acetylated is 22%. Actually, valid comparison can only be made upon the same time basis, since the acetyl derivative and free base have different rates of elimination and the proportion of conjugated amine appears larger, therefore, the greater the time which has elapsed since administration. The findings of Baines & Wien [1939] are not in harmony with the view expressed by Long & Feinstone [1938] that M and B 693 is less readily absorbed in man than is sulphanilamide.

The comparatively high M.L.D. of M and B 693 has been ascribed largely to incomplete absorption [Marshall *et al.* 1938]. Using the soluble sodium derivative, intravenously injected into dogs, Marshall & Long [1939] reported toxic

effects after 0·2 g./kg. with a blood level of 22 mg./100 ml. Deductions cannot be safely made concerning the toxicity of the free base, when administered orally, from experiments upon the intravenously injected sodium derivative. In the present work the sodium derivative of M and B 693 has been compared with the sodium derivative of sulphanilamide when both are administered orally. Acting largely upon Long's report [1939] upon "Sulfapyridine", the term adopted in America for 2-(*p*-aminobenzenesulphanilamido)pyridine, the Council on Pharmacy and Chemistry of the American Medical Association commented adversely upon the drug (see also *Lancet*, 1939, i, 703).

For the above reasons it was felt that a more extended comparison was desirable of the effects of M and B 693 and sulphanilamide, and of their respective sodium derivatives upon pigment metabolism.

Rats, in groups of 3, were again selected for the work and the experiments carried out upon the same general plan as those formerly reported [Rimington & Hemmings, 1939]. The dose of M and B 693 ranged from 0·2 to 2·9 g./kg. and that of the sodium derivative from 0·2 to 0·9 g./kg. All substances were administered daily *per os* (the sodium derivatives in aqueous solution, the insoluble M and B 693 as a suspension in gum acacia). The controls received the same quantity of gum acacia solution alone. Urinary porphyrin excretion was determined over 3-day intervals during a preliminary control period lasting about 12 days and dosing was then commenced.

Upon the completion of the observation period of 12–15 days, the rats of each group were killed, 4 hr. after receiving their last dose, and the concentrations of free and total amine in the blood of each of the three animals determined by Marshall's method, as used by Baines & Wien [1939]. Specimens were also taken for histological examination.

Results

The results are summarized in Table 1.

Table 1

Substance	Dose g./kg.	Blood level mg. per 100 ml. Free	Blood level mg. per 100 ml. Total	Porphyrin excretion × normal	Remarks
M and B 693	0·2	6·4	7·0	1·5	Loss of weight
		11·0	11·5		
		7·7	8·7		
	0·5	11·1	14·8	3·5	Loss of weight
		11·2	14·8		
	0·9	18·1	19·7	4	Marked loss of weight. One rat died on 15th day
		16·8	24·2		
		13·9	18·0		
	1·4	29	32	3	Bloods pooled before analysis
	2·9	38	41	5	Bloods pooled before analysis
M and B 693 Na derivative	0·2	—	—	No increase	Dosed for 12 days
	0·4	12·3	12·5	2	Same rats as above; dose raised to 0·4 g./kg. for 9 days. No loss of weight
		12·8	13·4		
		6·4	7·2		
	0·9	22·8	24·8	5·5	One rat died on 7th day. Bloods pooled before analysis
Sulphanilamide Na derivative	0·2	—	—	1·6	Dosed for 12 days
	0·4	10·8	13·2	2·4	Same rats as above; dose raised to 0·4 g./kg. for 9 days. No loss of weight
		4·7	6·5		
		5·0	6·8		

It will be seen from Table 1 that 0·2 g./kg. of M and B 693 daily for 12 days was practically without effect upon urinary porphyrin excretion, but that at higher dosage levels increases up to five times the normal quantity were recorded. In general, the severity of porphyrinuria developing corresponded to the blood concentration of the drug attained. Judged by this standard, the sodium derivative given by mouth appeared about as toxic as M and B 693 itself. The blood concentration was slightly lower at the lower dosage levels, due, no doubt, to more rapid elimination. Between the sodium derivatives of M and B 693 and of sulphanilamide there appears to be little, if any, difference, the latter compound producing slightly greater porphyrinuria. Considerable variation was noticed in drug levels in the bloods of different individual rats receiving the same dose, an observation in accordance with that of other workers. Nervous instability and restlessness were commonly observed in animals receiving the large doses of M and B 693. The urine of the group receiving 2·9 g./kg. contained haemoglobin from the 7th day onwards, and one rat dosed with 0·9 g./kg. of the sodium derivative died some hours after the 7th dose.

Histological examination of the livers, spleens and kidneys revealed congestion of the spleen in nearly all cases, but otherwise little evidence of abnormality. It is worthy of note that deposits of stainable iron in the spleens were only slightly increased, whereas such deposits are a characteristic feature after sulphanilamide administration.

Conclusions and summary

There is no doubt that M and B 693 administered to rats under the conditions of these experiments causes an increase in urinary porphyrin excretion. In this respect it resembles sulphanilamide and the other amines previously studied [Rimington & Hemmings, 1939]. Viewed from the quantitative standpoint, the degree of porphyrinuria developing as a response to dosage levels of 0·2–0·9 g./kg. is approximately the same for each substance, but whereas porphyrinuria increases at higher levels of sulphanilamide roughly in proportion to the dose, this relationship does not hold in the case of M and B 693. This may be due in part to the less complete absorption following larger doses, but, even in relation to the blood concentration levels, it appears that the porphyrinuria is relatively less marked at the higher than at the lower levels.

Whilst M and B 693 certainly possesses properties which render it potentially toxic, the study of its porphyrinuric action does not indicate that it is essentially any more toxic than is sulphanilamide in this same respect, even allowing for the less ready absorption of the pyridine derivative.

I am indebted to Mr A. W. Hemmings for his careful assistance in the care of the animals and in making the porphyrin determinations and to Mr T. A. Webster for the blood analyses. The drugs used in this work were kindly supplied to me by Dr Ewins of Messrs May and Baker, Ltd., and for this courtesy I wish to express my thanks.

REFERENCES

Baines & Wien (1939). *Quart. J. Pharm.* **12**, 4.
Hallam (1939). *Brit. med. J.* i, 559.
Ker-Gray (1939). *J.R. nav. med. Serv.* **25**, 422.
Long (1939). *J. Amer. med. Ass.* **112**, 538.
—— & Feinstone (1938). *Proc. Soc. exp. Biol., N.Y.*, **39**, 486.
Marshall, Bratton & Litchfield (1938). *Science*, **88**, 597.
—— & Long (1939). *J. Amer. med. Ass.* **112**, 1671.
Powell & Chen (1939). *J. Pharmacol.* **67**, 79.
Rimington (1939). *Proc. R. Soc. Med.* **32**, 1268.
—— & Hemmings (1939). *Biochem. J.* **33**, 960.
Stokinger (1939). *Proc. Soc. exp. Biol., N.Y.*, **40**, 61.
Wien (1938). *Quart. J. Pharm.* **11**, 217.

LINDA HALL LIBRARY
KANSAS CITY, MO.

15. BIOCHEMISTRY OF THE WOOD-ROTTING FUNGI

1. METABOLIC PRODUCTS OF *LENTINUS LEPIDEUS* FR.

By JOHN HOWARD BIRKINSHAW
and WALTER PHILIP KENNEDY FINDLAY

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London, and The Forest Products Research Laboratory, Department of Scientific and Industrial Research

(*Received 28 November 1939*)

The fungus *Lentinus lepideus* Fr. is a frequent cause of decay in worked timber, particularly in imperfectly creosoted material. It is often associated with the rotting of railway sleepers, telegraph poles and paving blocks, which it is able to attack on account of its tolerance of a comparatively high concentration of creosote. It is also an important factor in the decay of timber in mines. In culture it frequently produces well formed crystals in the medium or on the walls of the culture vessel and it has a strong, sweet, aromatic odour which is said to resemble that of Peru balsam. The present investigation was undertaken in order to identify the crystalline substance formed and if possible to determine the cause of the characteristic odour.

As the products under investigation were presumably volatile, the experimental material—Scots pine sapwood decayed by *L. lepideus*—was subjected to steam distillation. Only the matter volatile in steam was examined. The steam distillate deposited the crystals characteristic of the organism and the aqueous solution on extraction with ether afforded an oil having the characteristic odour. The crystalline substance was shown to be methyl *p*-methoxycinnamate. The oily extract was distilled *in vacuo* and the distillate was found to be a mixture of esters containing, in addition to methyl *p*-methoxycinnamate, esters of cinnamic and anisic acids. The cinnamic acid was present as the methyl ester since the distilled oil when treated with bromine gave the dibromide of methyl cinnamate. It was not possible to determine the alcohol associated with anisic acid owing to the small amount available. Methyl cinnamate has a sweetish aromatic odour not unlike that of *L. lepideus* and is no doubt responsible in part for the characteristic smell of the fungus.

The products isolated from *L. lepideus*, namely the methyl esters of cinnamic and *p*-methoxycinnamic acids and an ester of anisic acid, are structurally closely related and afford further examples of the production of methylated products by fungi. Many examples of methoxyl and methyl ester groups have already been noted amongst the products of the lower fungi.

The questions then arose as to (1) whether the products isolated are present in sound wood, (2) whether the esters described are characteristic of the organism and obtainable on other media which support growth of *L. lepideus* or whether they represent degradation products of some substance or substances present in wood alone and therefore obtainable by growth of the organism only on that medium.

Experiments were performed with the object of deciding these questions. Steam distillation of sound Scots pine sapwood afforded a volatile oil which could not be induced to yield crystals. Further, after saponification of the oil no trace of the acids obtainable after growth of *L. lepideus* could be detected. Hence it may be concluded that the esters identified do not pre-exist in the wood.

A culture of *L. lepideus* grown on malt agar was subjected to steam distillation. Although growth was poor and the examination of the culture was unavoidably delayed it was found that from the material volatile in steam it was possible to isolate a small amount of methyl *p*-methoxycinnamate which was satisfactorily identified. Thus this ester and probably the other esters formed by *L. lepideus* are characteristic products of the fungus and are not formed by degradation of wood alone.

It appears that methyl *p*-methoxycinnamate has not hitherto been recorded as occurring in nature, although the ethyl ester of *p*-methoxycinnamic acid was found by van Romburgh [1900, 1; 1902] to be the chief constituent of the essential oil of *Kaempferia galanga* Linné. *p*-Methoxycinnamic acid also occurs (in some form of combination) in "leptandra", the dried rhizome and roots of *Veronica virginica* Linné (*Leptandra virginica* Nuttall), a plant indigenous to the greater part of North America [Power & Rogerson, 1910]. "Leptandra" is used medicinally. The acid was obtained by cold alkaline hydrolysis of the chloroform extract of a resinous portion non-volatile in steam. It is not likely to have been present as the methyl ester, which is volatile in steam. Cinnamic acid was also present among the hydrolysis products of leptandra.

Cinnamic acid is found in nature in a wide variety of plant products, particularly in ethereal oils and resins. It is usually encountered in the form of esters. The methyl ester has been reported in the ethereal oil from the rhizome of *Alpinia malaccensis* [van Romburgh, 1900, 2] and in wartara oil [Schimmel & Co., 1901]. It is present to the extent of 48% in the root oil of *Alpinia galanga* [Ultée, 1911] and forms the chief constituent of the ethereal oil from the leaves of *Ocimum canum* Sims [Rouré-Bertrand Fils, 1914; Schimmel & Co., 1914]. Cinnamic acid or its esters are constituents of certain balsams. It occurs (chiefly as benzyl ester) in Peru balsam which *L. lepideus* is said to resemble in odour.

Anisic acid occurs in all ethereal oils which contain anethole from which it is produced by atmospheric oxidation. The occurrence in nature of esters of this acid does not appear to have been recorded.

As already stated *L. lepideus* is remarkably tolerant to relatively high concentrations of creosote and indeed is outstanding amongst the wood-rotting fungi in this respect. Thus it can attack wood which has been impregnated with solutions containing up to 4% of coal tar creosote, i.e. which contains up to 15 kg. of creosote per cubic metre of wood. There may be some connexion between this property of the fungus and its power to elaborate aromatic substances of the type which have been isolated.

It is possible that what we may term the "benzenoid environment" due to the metabolic products described has rendered the fungus immune to the toxic action usually associated with such substances and hence resistant to certain constituents of creosote itself. It is noteworthy, however, that two of the acids formed, namely *p*-methoxycinnamic and anisic acids, are methoxyl derivatives of phenolic acids. It is recognized that methylation is a method employed by nature, particularly in the plant kingdom, for detoxicating substances which would prove harmful to the organism. It may be that the phenolic acids or their esters are the primary metabolic products of the fungus and that these

are then methylated and so rendered harmless. Granted the power of achieving this methylation of metabolic products, the fungus may apply the same method to the phenolic constituents in creosote which are presumably the most active fungicidal agents present and thus effect at any rate a partial detoxication. We have at present no evidence as to whether either of these hypotheses is correct.

Experimental

Organism

A culture of *Lentinus lepideus*, which was originally isolated by Dr Liese at the Forestry School, Eberswalde, Germany, was used. This particular isolation was chosen because it appeared to form crystals in culture more readily than any of the other strains available. It may be noted that this isolation is the one which it has been agreed to use for standard laboratory tests on the toxicity of wood preservatives.

Method of culture

The fungus was grown on 5% malt agar in special Kolle flasks. After the surface of the agar was covered with mycelium four blocks of Scots pine sapwood measuring $5 \times 2{\cdot}5 \times 1{\cdot}5$ cm., which had been sterilized in an autoclave at 15 lb. pressure, were introduced into each flask. These were supported on low glass rests so that they came into contact only with the mycelium and did not touch the agar. The flasks containing the sample blocks were then kept in an incubator at 22° for 3 months, and at the end of this period the blocks were removed from the flasks and submitted to analysis.

Since it was considered undesirable to oven-dry the blocks before the experiment, their original oven-dry weight was estimated by drying a large number of similar blocks of the same size which had been cut from the same pieces of wood. In this way the average oven-dry weight of a single sample block was found to be 7·93 g.

Treatment of infected wood

The Scots pine sapwood (118 blocks) which had been inoculated with *L. lepideus* was placed in a 5 l. flask and subjected to steam distillation for 6 working days. From the results of previous experiments it was estimated that the blocks had lost about 20% of their oven-dry weight as a result of the decay brought about by this fungus during 3 months at 22°, so that the dry weight of material used for analysis was estimated at approximately 748 g. Crystals separated in the distillate and were collected after chilling. The yield decreased each day, that obtained on the 6th day being only 0·06 g. The total yield of crude crystalline material (A) was 1·29 g., M.P. 85·5–86·5°.

The filtrates from the crystals were extracted with ether. The liquid extract remaining after removal of ether had the characteristic sweet ethereal smell of *L. lepideus*. This residue (wt. 1·62 g.) was distilled at 2–3 mm., yielding fraction I (B), B.P. 94–110°, wt. 0·83 g. clear yellowish oil, and fraction II, B.P. 110–122°, wt. 0·23 g. pale yellow cryst. solid M.P. 70–85°.

The residue in the distillation flask had darkened considerably and finally became a black tarry mass.

Fraction II on recrystallization from 1 ml. of methyl alcohol gave 0·18 g. of material melting at 87–89° and identical with the crystalline material (A) obtained directly from the steam distillate by filtration.

Examination of the crystalline material (A)

The crude material, M.P. 85·5–86·5°, was recrystallized from methyl alcohol and then had M.P. 88°. (Found: C, 68·87, 68·80; H, 6·19, 6·36; OMe, 30·8, 30·8%. N, zero. Mol. wt. (Rast camphor method), 204, 206. $C_{11}H_{12}O_3$ (with 2OMe) requires C, 68·74; H, 6·30; OMe, 32·29%. Mol. wt. 192.)

Saponification. Substance A was insoluble in cold aqueous NaOH. It was hydrolysed by heating 0·2 g. with 5 ml. of *N* methyl alcoholic KOH for 30 min. under reflux. The solid first went into solution, then crystals of the K salt separated. These were dissolved by addition of about 25 ml. of water and the mixture was acidified with HCl. The precipitated acid was collected and dried *in vacuo*—wt. 0·16 g., M.P. 168–170°. On recrystallization from benzene it gave needles of M.P. 172–173°. The melt was cloudy but it cleared at 187°. Sublimation *in vacuo* did not further raise the M.P. (Found: C, 67·45; H, 5·47; OMe, 17·2%. Equiv. by micro-titration, 180·6. $C_{10}H_{10}O_3$ (1OMe) requires C, 67·41; H, 5·66; OMe, 17·4%. Equiv. (for monobasic acid), 178·2.)

Substance A is evidently the monomethyl ester of an acid $C_{10}H_{10}O_3$ with one methoxyl group.

Action of $KMnO_4$ on substance A. The substance (0·19 g.) was dissolved in 50 ml. of pure acetone and finely ground $KMnO_4$ was added in small portions with shaking. As the solution became slightly warm it was cooled under the tap. After 0·6 g. of $KMnO_4$ had been added the oxidation became very slow. The solution was filtered and the MnO_2 was washed with acetone and dried. It was then extracted with water and the clear filtrate was acidified with HCl. The precipitated acid was collected, washed and dried. It weighed 0·095 g., M.P. 181–183°. The M.P. after recrystallization from water was raised to 182–183°. A further yield was obtained from the acetone by continuing the addition of $KMnO_4$. (Found: C, 63·55; H, 5·42; OMe, 20·2%. Equiv. by titration, 151·2. $C_8H_8O_3$ (1OMe) requires C, 63·17; H, 5·30; OMe, 20·4%. Equiv. (as monobasic acid), 152·1.)

This substance obtained by oxidation proved to be anisic acid since it showed no depression in M.P. when mixed with an authentic specimen of that substance. The saponification acid would therefore be *p*-methoxycinnamic acid, confirmed by the fact that it gave no depression in M.P. when mixed with authentic material synthesized for comparison by the method of Knoevenagel [1898]. The synthetic acid had the same M.P. 172–173° and likewise gave a turbid fluid on melting which became clear at 188°. The original substance A is thus methyl *p*-methoxycinnamate. It showed no depression in M.P. when mixed with the synthetic ester of the same M.P.

Examination of the distillate B

The mol. wt. (Rast) of the liquid distillate in two determinations was 159 and 150. The methoxyl content by Zeisel's method was 24·5%. As it seemed probable that it was also an ester it was subjected to alkaline hydrolysis.

Saponification by cold methyl alcoholic KOH. The substance (0·1046 g.) was treated with 2·00 ml. of approximately *N* KOH in MeOH and 1 ml. of MeOH was added. It was left stoppered at room temperature for 3 days and then titrated, a control (without substance) receiving similar treatment. The titration value of the acid produced was 0·520 ml. giving an apparent mol. equiv. of 201 for the original substance. The neutralized solution was diluted with water and acidified. An acid was precipitated which was separated after chilling the solution. The crystalline acid (0·03 g.) had M.P. 97–110°. On recrystallization

from light petroleum (B.P. 60–80°) it had M.P. 124–127·5° and equiv. by micro-titration of 145. The amount obtained was too small for further recrystallization and as from the molecular equiv. the hydrolysis appeared to have been incomplete another hydrolysis was undertaken on a fresh portion using aqueous NaOH.

The liquid (0·1934 g.) was treated with 5 ml. of aqueous *N* NaOH. It gave a bright golden yellow colour. After 1·5 hr. under reflux all the oil had gone into solution. Titration now gave an apparent equiv. for the original substance of 160. The acid (0·12 g.) was precipitated with HCl and collected. It had M.P. 110–126° but proved even less amenable to purification by crystallization from light petroleum than the preceding sample, whereas it was too soluble in other solvents. The acids were converted into *p*-nitrobenzyl esters, but these also proved difficult to fractionate by crystallization. Accordingly it was decided to revert to the method of saponification with methyl alcoholic KOH in the cold, which, although not proceeding to completion, gave a purer product.

The oil (0·2503 g.) was treated with 5 ml. of *N* methyl alcoholic KOH and a further 2 ml. of methyl alcohol were added. The mixture was left for 3 days at room temperature. The solution was deep reddish brown immediately after addition of the KOH solution, but lightened on keeping. Titration gave an apparent mol. equiv. of 199, showing that again hydrolysis was not complete. The acids were precipitated as an emulsion which crystallized on chilling. The crystals were collected and dried. They weighed 0·095 g. and had M.P. 100–103° after softening at 92°. After seven recrystallizations from light petroleum during which the M.P. was gradually raised to 129–130°, the weight was only 7 mg. When mixed with pure cinnamic acid of M.P. 133°, the M.P. was not depressed. (Found: C, 73·46; H, 5·52%. Calc. for cinnamic acid ($C_9H_8O_2$), C, 72·95; H, 5·44%.)

The mother liquor from the final crystallization of the above acid was evaporated and the crystals were dissolved in about 2 ml. of carbon disulphide and treated with minute drops of bromine until the colour of the bromine persisted. During this treatment crystals separated. These were collected, recrystallized from light petroleum (B.P. 50–60°) and then from chloroform. There was considerable loss from the latter solvent. A synthetic sample of dibromide was prepared in similar manner from cinnamic acid. The two specimens were heated side by side together with a mixture of the two. All melted together at 200–201° with some decomposition. Thus the product obtained by the action of bromine is undoubtedly cinnamic acid dibromide, confirming the presence of cinnamic acid in the hydrolysate.

Further examination of the hydrolysis products of liquid B

The whole of the remaining material from the mother liquors of crystallization of cinnamic acid and the ether extract of the aqueous filtrate obtained after the initial precipitation with HCl were combined and further hydrolysed with aqueous NaOH. The material (0·22 g.) was treated with 5 ml. of *N* NaOH and boiled for 1·5 hr. under reflux. A back titration showed that organic acid equivalent to 1·43 ml. of *N* alkali was present. The acids were precipitated with HCl and collected (weight 0·11 g.).

The acids were heated under reflux with about 15 ml. of light petroleum (B.P. 50–60°). Some remained undissolved. The mixture was filtered and the undissolved material, M.P. 160–175°, recrystallized from benzene. It now had M.P. 178–180°, raised by recrystallization from water to 180–182°. Mixed with

authentic anisic acid of M.P. 183°, the M.P. was 182–183°. Hence the product is anisic acid. (Found: C, 63·44; H, 5·31%. Calc. for anisic acid ($C_8H_8O_3$), C, 63·17; H, 5·30%.)

Preparation of the dibromide of methyl cinnamate from product B

Product B (0·19 g.) in about 2 ml. of carbon disulphide was treated with minute drops of bromine until the latter was no longer decolorized. There was an evolution of HBr which should not occur with mere addition of bromine, probably due to some unidentified product in the mixture of esters. The carbon disulphide was removed *in vacuo* leaving a mixture of crystals and oil. The crystals were washed with a little ethyl alcohol to remove the oil and drained. The colourless compact prisms (0·15 g.) so obtained had M.P. 114–115·5°, raised on recrystallization from ethyl alcohol to 116·5–118°. Yield 0·10 g. When mixed with synthetic material of the same M.P., prepared by addition of bromine to methyl cinnamate, there was no depression. (Found: Br, 50·04, 50·11%. Calc. for $C_{10}H_{10}O_2Br_2$, Br, 49·64%.)

This affords conclusive evidence that the cinnamic acid occurs as the methyl ester. The alcohol associated with the anisic acid has not been determined. It seems likely that the anisic acid ester will prove to be methyl anisate, however, since the other two acids identified are present as methyl esters. This also accords with the mol. wt. of the mixed liquid esters (product B), namely 159, 150.

Examination of volatile products of L. lepideus *grown on malt agar*

The fungus was cultivated on a layer of malt agar (5%) in Kolle flasks. After 10 weeks' incubation at 22° the flasks were placed in the refrigerator to await examination, which took place 4 weeks later. The growth was poor and, owing to the delay in examination, only small yields of products were expected. The whole of the material, fungus and agar, was subjected to steam distillation. The first distillate (1800 ml.) did not deposit crystals on chilling so it was extracted with ether. After evaporation of the ether there remained a small amount of sticky crystals (0·03 g.). Further steam distillation gave only 0·01 g. of material. The combined ether extracts were subjected to vacuum sublimation from a bath at 40°. The crystals obtained had M.P. 73–77° raised by second and third sublimations to 84–86·5 and 86–88° respectively. The purified product showed no depression in M.P. when mixed with authentic methyl *p*-methoxycinnamate. Thus it is evident that methyl *p*-methoxycinnamate is produced by *L. lepideus* on media other than wood. Owing to the small amount of product obtained on steam distillation no attempt was made to identify other esters formed.

Volatile products from wood

743 g. of fresh chips of Scots pine sapwood were subjected to steam distillation. Ether extraction of the distillate gave an oil (1·2 g.) which showed no sign of crystals. This was distilled *in vacuo* at 1–2 mm. and gave two fractions: I, B.P. 55–75°, 0·72 g.; II, B.P. 75–86°, 0·23 g. Both fractions were clear yellow oils which did not crystallize.

As it was thought that traces of the products sought would be more readily obtained crystalline in the form of free acids, both oily fractions were subjected to alkaline hydrolysis by boiling with 20 ml. of *N* NaOH for 2·5 hr. The unsaponifiable matter was then extracted with ether and the aqueous portion acidified with HCl. No crystals were obtained on acidification and ether extraction of the acids gave an oil in which there was only a trace of doubtfully

crystalline material. This portion was subjected to vacuum sublimation but no solid, only an oil, was obtained. Thus it appears that the identified products obtained from cultures of *L. lepideus* do not pre-exist in the wood. They are therefore true metabolic products of the fungus.

SUMMARY

The wood-rotting fungus *Lentinus lepideus* Fr., when grown on Scots pine sapwood, gives rise to volatile metabolic products. Amongst these methyl *p*-methoxycinnamate, methyl cinnamate and an ester of anisic acid (probably the methyl ester) have been detected. These products are shown to be absent from uninfected wood. Methyl-*p*-methoxycinnamate has been identified amongst the products obtained by growth of *L. lepideus* on a malt-agar medium.

We wish to express our appreciation of the interest shown in this work by Prof. H. Raistrick and to thank Mr A. Bracken for technical assistance.

REFERENCES

Knoevenagel (1898). *Ber. dtsch. chem. Ges.* **31**, 2596.
Power & Rogerson (1910). *J. chem. Soc.* **97**, 1944.
van Romburgh (1900, 1). *Proc. Acad. Sci. Amst.* **3**, 38.
—— (1900, 2). *Proc. Acad. Sci. Amst. Wisk. en Naturk. Afd.* p. 445.
—— (1902). *Proc. Acad. Sci. Amst.* **4**, 618.
Rouré-Bertrand Fils (1914). *Chem. Zbl.* 1, 542.
Schimmel & Co. (1901). *Ber. dtsch. chem. Ges.* April, 62; *Chem. Zbl.* **1**, 1008.
—— (1914). *Chem. Zbl.* **1**, 1654.
Ultée (1911). Cf. Schimmel & Co. (1911), *Chem. Zbl.* 1, 1837.

16. HISTIDINE DETECTION AND ESTIMATION IN URINE

By E. RACKER

From the Biochemical Laboratory, Cardiff City Mental Hospital

(*Received 20 November 1939*)

During the last 10 years many attempts have been made to detect changes in the liver metabolism of patients suffering from mental disorders. For example, in this hospital investigations have been carried out which indicate changes in the sugar tolerance of manic depressive patients [McCowan & Quastel, 1931], while diminution of hippuric acid excretion after benzoate administration has been shown by Quastel & Wales [1938] to take place in catatonic patients.

Schimmelpfeng [1937] stated that there is an increased histidine excretion in melancholic patients and he correlated this with disturbances of the liver function.

The object of this work was to try to confirm and to follow up this statement of Schimmelpfeng.

Detection and estimation of histidine

Histidine was first isolated by Engeland [1908] in very small quantities from 40 l. of normal urine. Hunter [1922] isolated 0·1 g. histidine from 1 l. of the urine of a patient suffering from measles. Armstrong & Walker [1932] isolated from 10 l. of pregnancy urine 0·2 g. of histidine monohydrochloride and stated that there was much loss during the isolation.

For the detection of histidine in urine two colour tests have been evolved. Firstly there is the diazo reaction of Pauly [1904], a modification of which by Koessler & Hanke [1919; 1924] is generally used. However, this reaction is not considered to be specific because, as stated by Koessler & Hanke [1919], Hunter [1925] and others, iminazoles, certain purines, phenols, urine pigments and tyrosine give this reaction as well as histidine. Therefore they interfere with a quantitative determination of the latter. Again, this reaction is very easily interfered with by small amounts of metallic salts, e.g. copper, cobalt; also by some amino-acids, alcohols and uric acid. All these facts render the application of this very sensitive method unsuitable for a quantitative estimation of histidine in urine.

The second colour test used for histidine is the bromine reaction first described by Knoop [1908]. Hunter [1922] and Kapeller-Adler [1933] have modified this test and have increased its sensitivity. Since this test is considered by many authors [Knoop, 1908; Hoppe-Seyler & Tierfelder, 1924; Armstrong & Walker, 1932; Kapeller-Adler, 1936; Tschopp & Tschopp, 1938] as specific for histidine attempts have been made by many workers to apply it to human urine especially since Voge [1929] and Kapeller-Adler [1933] suggested it as a means for the early detection of pregnancy. Many contradictory results have been published since then about histidine excretion in urine. Tschopp & Tschopp [1938], considering these results as well as those of their own investigations, came to the conclusion that histidine is excreted sometimes in normal but more frequently in some pathological conditions. This was especially so in liver diseases and allergic conditions. They also used the bromine test as described by Kapeller-Adler [1936].

In principle the test, as applied qualitatively by Kapeller-Adler [1936], Schimmelpfeng [1937], Tschopp & Tschopp [1938] and many others, is as follows:

To 5 ml. of urine are added drop by drop the bromine reagent until a slight excess of bromine is indicated by starch-iodide paper. After 10 min. ammonia-ammonium carbonate solution is added, then the test tube is placed in boiling water for a short period. A red-violet colour indicates the presence of histidine.

The disadvantages of this method are as follows: In the first place the pigmentation of the urine renders the value of the colour test sometimes rather uncertain. In the second place the development of the colour is often inhibited by precipitation of phosphates after the alkali treatment. This was already observed by Kapeller-Adler [1933]. Addition of fairly large quantities of histidine to urines rich in phosphates quite often did not give a corresponding colour reaction. Thirdly there are substances in urine which sometimes greatly increase the colour produced by bromination.

This was proved by experiments on specially treated normal urines. The phosphates were first removed with baryta. The *p*H was adjusted to 7·4, then all pigments and finally all the histidine were removed with animal charcoal. After readjusting the *p*H to that of a standard histidine solution, an equal amount of histidine was added to each sample. Application of the bromine test resulted generally in an increased depth of colour and often in a different shade from that given by the standard. The increase of colour varied from sample to sample.

Effect of urates on the bromine colour test

On further investigation of the above phenomenon it was found that the addition of urates to a pure histidine solution greatly increased the sensitivity of the colour reaction and also affected the colour itself.

Since urines contain different quantities of urates and other sensitizing agents, different samples of urines containing even the same quantity of histidine may show different intensities of colour.

Effect of substances other than urates on the bromine test

It was found in the case of patients who had had somnifaine[1] treatment, that the apparent histidine excretion was high. Investigation showed that this apparent increase was due to the excretion of somnifaine, which itself has an intensifying action on the bromine colour test. Thus the property of urates in intensifying the colour test is shared by other molecules, in particular by some unsaturated fatty acids and unsaturated alcohols. (The sensitizing agent in somnifaine is the unsaturated molecule allyl*iso*propylbarbituric acid.)

It was then decided to modify the existing method of histidine detection by adding to all urines an excess of urates. This procedure resulted in the elimination of all differences in intensity and shade of the colour reaction due to the presence of varying quantities of sensitizing agents in the urines, and thus made quantitative estimation of histidine possible.

Method

Chemicals required

(1) Histidine standard solution. 100 mg. histidine monohydrochloride are dissolved in 1 l. of $N/10$ H_2SO_4.

(2) 5% sodium urate suspension. 25 g. sodium urate are added to 500 ml. of distilled water. This is left to stand for 1 day. The suspension is shaken before use.

[1] Somnifaine is a narcotic containing diethylbarbituric acid and allyl*iso*propylbarbituric acid.

(3) Bromine-acetic acid solution. 2 ml. of bromine are added to 100 ml. of glacial acetic acid, then made up to 300 ml. with distilled water.

(4) Arsenious oxide solution. This is a saturated solution of As_2O_3 in a 10% NH_3.

(5) Na_2CO_3 (anhydrous).

(6) Saturated baryta.

(7) *N* H_2SO_4.

(8) Animal charcoal purified (for decolorizing purposes).

Procedure

(1) 6 ml. of urine are pipetted into a test tube and 3 ml. of baryta added. The mixture is now filtered.

(2) To the filtrate from above 3 ml. of *N* H_2SO_4 and about 100 mg. of charcoal are added. The mixture is again filtered.

(3) 6 ml. of the clear, colourless filtrate and also 6 ml. of the histidine standard are each treated with 2·5 ml. urate suspension. Bromine in small portions is added until a slight excess is indicated by a faint persistent yellow colour.

(4) The solutions are kept for 10 min. with occasional shaking. The excess of bromine is removed by 1–2 drops of arsenious oxide, when the solutions should again become colourless. If this is not so, the procedure up to this point should be repeated with a fresh sample of urine, using a little more charcoal.

(5) The test tubes are placed in boiling water and, in order to prevent excess of frothing and of dilution, small quantities of solid Na_2CO_3 are added until no more CO_2 bubbles are produced. The test tubes are shaken from time to time. 5–7 min. are required to complete the reaction.

(6) The test tubes are taken from the water bath and after standing 10 min. the orange-red colours are compared in the colorimeter. The values thus obtained for the amount of histidine in the urine must be multiplied by 2, because to the 6 ml. of urine 3 ml. of baryta and 3 ml. of H_2SO_4 were added.

Rationale of the method

For reasons already stated the phosphates are removed with baryta. The excess of baryta is removed and the solution brought to an acidity roughly corresponding to the *p*H of the standard. It has been found that charcoal, when added in small quantities to a solution of this acidity, absorbs histidine to a negligible extent. The actual amount of charcoal to be used depends on the activity of the charcoal sample. The smallest possible amount resulting in a colourless filtrate is taken.

The use of arsenious oxide is recommended by Conrad & Berg [1937]. It must be added since the presence of an excess of bromine considerably diminishes the colour. Kapeller-Adler suggested that this is due to a further attack of the histidine molecule by the excess of bromine. However, Conrad & Berg [1937] and Woolley & Peterson [1937] have shown that if the excess of bromine is removed before the alkali is added the colour development is not inhibited. Hence it seems improbable that much of the histidine is further attacked during the 10 min. contact with the bromine. Woolley & Peterson therefore supposed that the histidine is not attacked by bromine itself, but that conversion of histidine into the corresponding aldehyde takes place by the action of NH_4OBr, which is produced by the addition af ammonia. It is more probable, however, that this inhibition is due to the fact that bromine oxidizes the molecule responsible for

the colour. This was shown by addition of bromine after the colour had already completely developed. Similarly decoloration took place with oxidizing agents such as H_2O_2.

Sensitivity of the method and experimental error

The sensitivity of the bromine reaction, which is described by Hunter [1922] as 1 : 10,000 and by Tschopp & Tschopp [1938] as 1 : 6250, is increased by this modification to 1 : 100,000.

The experimental error is about 10 % according to controls in which known quantities of histidine have been added to the urine (see Table 1).

Table 1

Case	Histidine originally in urine mg./100 ml.	Histidine added to 6 ml. urine mg.	Histidine calculated mg./100 ml.	Histidine estimated mg./100 ml.	Experimental error %
M. N.	9·1	0·8	22·4	21·6	−3·5
F. T.	17·3	0·8	30·6	27·9	−8·8
R. E.	75·5	1·0	92·1	90·3	−1·9
D. J.	20·3	1·3	42·0	38·8	−7·6

Results

More than 400 samples of urine of some 180 persons have been examined. Morning urine or 24 hr. specimens have been used. It has been found that in the urines of normal persons as well as in those of all kinds of mental disorders so far investigated the bromine test proved to be positive. The colours obtained by this test were on an average equivalent to that of a 20 mg./100 ml. histidine monohydrochloride solution. However, the figures varied between 2 and 80 mg./100 ml. (see Table 2 for typical results).

Table 2. *Results of normal cases and patients with mental disorder*

Case	Sex	Diagnosis	Histidine mg./100 ml.	Urea g./100 ml.
D. W.	F.	Normal	19	1·6
D. J.	M.	Normal	21	2·1
G. J. J.	M.	Normal	64	—
A. S.	F.	Normal	6	0·9
R. E.	M.	Normal	75	2·3
W. H.	M.	Melancholia and arteriosclerosis	36	1·9
C. D.	F.	Melancholia	18	1·3
L. A. D.	M.	Melancholia	10	1·1
H. A.	F.	Agitated melancholia	23	—
W. D.	F.	Melancholia and diabetes	12	—
Ll.	M.	Melancholia	50	—
A. M.	F.	Dementia paranoides	17	1·6
S. R. (negro)	M.	Schizophrenia	51	—
O. R.	M.	Schizophrenia	33	1·7
S. M.	F.	Schizophrenia	11	—
R. J.	F.	Senile dementia	7	0·9
S. M.	F.	Mania	23	2·0
M. S.	F.	Primary dementia (jaundice)	48	—

A proportionality between histidine and urea excretions has been observed as well as a certain proportionality to the specific gravity. But this does not account for the large individual differences. In three cases the histidine excretion was followed up for 14 days and compared with the urea excretion. The examinations were carried out twice daily in the first week, the first time on the morning urine, the second time 2 hr. after lunch.

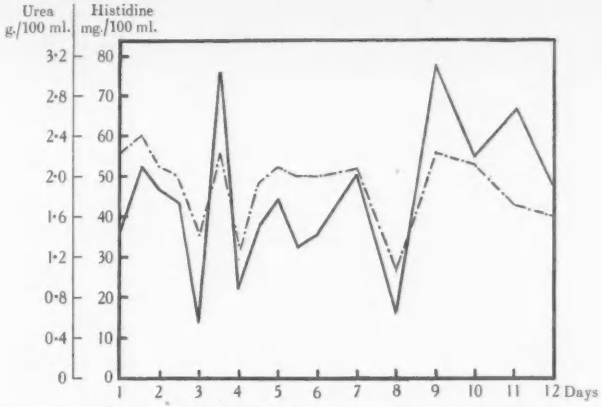


Fig. 1. Normal case. Histidine excretion ——; urea excretion —·—

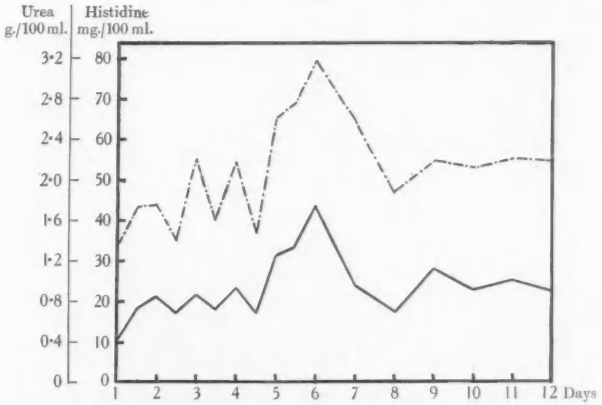


Fig. 2. Normal case. Histidine excretion ——; urea excretion —·—

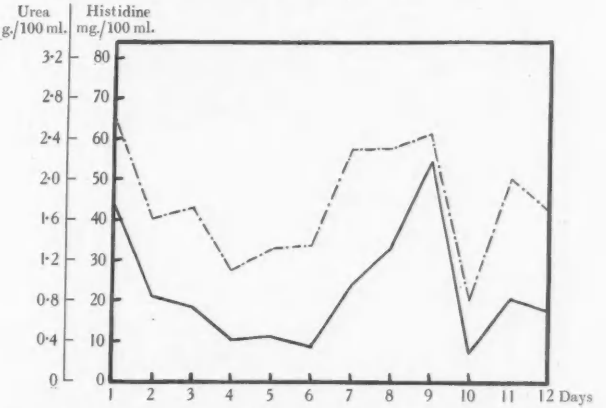


Fig. 3. Melancholic patient. Histidine excretion ——; urea excretion —·—

The first two of these persons were normal and the third was a melancholic patient. The results are shown in Figs. 1–3.

These results do not support the conclusion of Schimmelpfeng [1937] that melancholic patients excrete abnormally large quantities of histidine.

Results of pregnant cases

The urines of 27 pregnant women have been investigated. Six of these urines showed definitely high values (Table 3).

Table 3

Case	Age years	Duration of pregnancy months	Histidine mg./100 ml.	Urea gm./100 ml.	Specific gravity
A. S.	23	6	162	1·4	1018
T. M.	20	6	109	1·3	1022
K.	24	4	97	1·4	1024
M. C.	28	3	97	1·4	1023
G. L.	30	3	96	2·0	1023
J. D.	?	3	107	1·3	1018

Although the other urines showed figures within the limit of normal values, it must be noted that the urea content and the specific gravity of these urines were extremely low (see Table 4).

Table 4

Case	Age years	Duration of pregnancy month	Histidine mg./100 ml.	Urea g./100 ml.	Specific gravity
L. P.	22	6	39	0·9	1012
T. B.	?	3	44	0·7	1013
M. M.	37	4	63	1·3	1015
S. M.	28	3	12	0·4	1006
W. McD.	—	2	27	0·5	1007
Non-pregnant women:					
A. H.	56	—	28	2·2	1021
K. F.	37	—	56	2·6	1027
M. S.	27	—	10	1·5	1015

If the proportion of histidine to urea content and to specific gravity is taken into account the histidine excretion must be regarded as high. For the possible explanation of this phenomenon attention may be drawn to the investigations of Kapeller-Adler & Herrmann [1936] and Kapeller-Adler & Boxer [1937] in which they claim to have shown that gonadotropic hormones inhibit the action of histidase in liver brei. Also the urines of pregnant women treated by the alcohol precipitation method (Zondek) are stated to have a similar inhibitory action presumably due to the gonadotropic hormones present.

Discussion of the specificity of the bromine test

Knoop [1908], after examining a large number of substances with his test, concluded that only histamine besides histidine gave a positive reaction. Linneweh *et al.* [1929] stated that *N*-methylhistidine gave this reaction as well, and Kapeller-Adler confirmed this, but observed that only a fifth of the colour was obtained. Armstrong & Walker [1932] considered Knoop's test as specific for histidine and *N*-methylhistidine, although they mentioned that "a few substances have been met, which gave with bromine water a reaction of a

different type". Thus they described a reaction of bromine water with adrenaline and tyramine. With regard to histamine, they stated that the colour obtained can be extracted with amyl alcohol. Woolley & Peterson [1937] described a few substances which interfere with the histidine determination by the bromine reaction. They observed a brown shade if substances such as tyrosine and tryptophan were present.

These results can be confirmed in general. A number of other substances were found to react with bromine; some phenols (e.g. aminophenol), some alkaloids (e.g. morphine), tyrosine and tryptophan. The last two were mentioned by Woolley & Peterson as interfering substances. It was, however, found that they gave a reaction by themselves.

All these substances showed more or less marked differences from histidine in reacting with bromine. Some of them, such as morphine, were not decolorized by the addition of arsenious oxide after bromination. A yellow colour remained which was proportional to the amount of morphine present. The addition of sodium carbonate in the cold gave a reddish colour, some of which was readily extractable by amyl alcohol from the alkaline solution. This was not the case with histidine. The colour test for all substances except histidine mentioned above is characterized by this important difference. With regard to histamine it must be noted that a much less intense colour and a different shade are obtained by the modified bromine method.

Hence all substances investigated so far could be distinguished from histidine. The substance in urine giving the bromine reaction showed in every respect the characteristics of histidine. But considering the various kind of substances which have been found to give colour reactions with bromine, it is felt that this reaction may not be as completely specific for histidine as would appear from the literature. It is therefore possible that the unexpected high figures of histidine excretion in normal urine may perhaps be partly due to another substance the reaction of which with bromine has not yet been investigated.

Experiments are therefore in progress to isolate the histidine from urine, using a new method by which it is hoped large losses will be avoided.

Summary

1. The application of present colorimetric methods for histidine detection and their difficulties are described.

2. A simple and rapid quantitative method for histidine estimation in urine by a modification of Knoop's bromine reaction is given.

3. The specificity of the bromine test is discussed.

4. When this reaction is applied to normal urine a colour is produced which corresponds to an average of 20 mg./100 ml. histidine monohydrochloride. The range of normal values lies between 2 mg./100 ml. and 80 mg./100 ml.

5. A higher excretion value in the urines of melancholic patients, as claimed by Schimmelpfeng, could not be confirmed.

6. Pregnancy urines usually show high histidine values when considered in relation to the urea excretion and to the specific gravity of the urine.

The author would like to express his gratitude to Dr J. H. Quastel for the privilege of working in his department and for his help and advice throughout this work. He is also grateful to the Medical Superintendent of this Hospital for permission to work with the patients, and to Dr H. A. Scholberg for his kindness in procuring pregnancy urines.

REFERENCES

Armstrong & Walker (1932). *Biochem. J.* **26**, 143.
Conrad & Berg (1937). *J. biol. Chem.* **117**, 351.
Engeland (1908). *Hoppe-Seyl. Z.* **57**, 49.
Hoppe-Seyler & Tierfelder (1924). Handb. d. physiol. u. path. Chem. Analyse.
Hunter (1922). *Biochem. J.* **16**, 637.
—— (1925). *Biochem. J.* **19**, 25.
Kapeller-Adler (1933). *Biochem. Z.* **264**, 131.
—— (1936). *Klin. Wschr.* **11**, 1728.
—— & Boxer (1937). *Biochem. Z.* **293**, 207.
—— & Herrmann (1936). *Klin. Wschr.* **15**, 977.
Knoop (1908). *Beitr. chem. Physiol. Path.* **11**, 396.
Koessler & Hanke (1919). *J. biol. Chem.* **39**, 497.
—— —— (1924). *J. biol. Chem.* **59**, 803.
Linneweh, Keil & Hoppe-Seyler (1929). *Hoppe-Seyl. Z.* **183**, 11.
McCowan & Quastel (1931). *Lancet*, ii, 731.
Pauly (1904). *Hoppe-Seyl. Z.* **42**, 508.
Quastel & Wales (1938). *Lancet*, p. 301.
Schimmelpfeng (1937). *Klin. Wschr.* **11**, 1567.
Tschopp & Tschopp (1938). *Biochem. Z.* **298**, 206.
Voge (1929). *Brit. med. J.* (ii), 289.
Woolley & Peterson (1937). *J. biol. Chem.* **122**, 207.

17. THE EFFECT OF ACUTE MAGNESIUM DEFICIENCY ON BONE FORMATION IN RATS

By JOHN DUCKWORTH, WILLIAM GODDEN and GRACE McMARTIN WARNOCK

From the Rowett Institute, Aberdeen

(*Received 24 November 1939*)

The large skeletal requirements for Ca and P and the importance of correct amounts and balance of these elements in diets have resulted in much greater attention being given to their metabolism than to the metabolism of Mg. The amount of Mg in bone is only one-fortieth to one-fiftieth of that of Ca and this low proportion has tended to direct attention away from Mg when consideration is given to the composition and formation of bone salt. Within the last few years increasing attention has been given to Mg and improvements in analytical technique and in knowledge of the preparation of synthetic diets have been responsible for certain advances.

The crystallographic studies of Gruner *et al.* [1937] on enamel and dentine have indicated that atomic replacements can take place in such crystal structures. If their conclusions can be extended to bone salt, the manner in which this material can serve as a reserve of mobilizable Mg could be explained as an exchange or replacement process. That bone salt does act as such a reserve is not yet certain. While some of the evidence favours such a view, contradictions exist. The extent to which bone salt can act as a Mg reserve, that is to say the extent to which replacement can occur in cases where the organism is compelled to draw on the reserve owing to a dietary deficiency of Mg, is so far unknown. The relation between Mg and bone formation, as distinct from its probable presence in the bone salt molecule, has been investigated only to a slight extent and, beyond the accelerating effect of Mg upon the hydrolytic action of phosphatase *in vitro*, very little has been established.

The present communication is concerned with a portion of this problem and studies in acute deficiency have been the method of approach.

Some aspects of Mg metabolism have been reviewed elsewhere by one of us [Duckworth, 1938–9] and only those certain studies of bone formation on acutely deficient diets will be referred to during the discussion of the present results.

Experimental

Preparation of the Mg-deficient diet. One of the principal difficulties in this investigation has been the preparation of a diet which, while adequate in respect of all other known dietary factors, should have a minimal Mg content. This procedure has involved processes of purification designed to remove Mg from all the components of the diet; difficulties arose particularly with respect to adequate supplies of the vitamin B complex. Sources of this vitamin are frequently found to contain relatively large amounts of Mg and it was feared that any process for removal of the Mg might result in a loss of vitamin B potency. Accordingly, two sources of vitamin B complex were used, namely, yeast and liver extract, and as an added safeguard the extracts were prepared

and the Mg was removed by two somewhat different processes. The purified vitamin B extracts were fed at a fairly high level to ensure completeness of the diet and this resulted in a somewhat higher Mg content than would otherwise have been necessary. The complete Mg-deficient diet was found by spectrographic analysis[1] to contain not more than 0·0006 % Mg (i.e. 6 parts per million).

Preparation and purification of yeast extract. To 300 g. dried brewer's yeast (obtained from the Pharmaco-Chemical Products Co., Ltd., Burton-on-Trent), 1200 ml. of 50 % C_2H_5OH were added and the mixture was vigorously shaken for 15 min. and filtered on a Büchner funnel. The yeast was extracted twice more in the same manner except that 900 ml. of alcohol were used in each case. The three filtrates were combined and the volume measured (generally about 2700 ml.). About 15 g. of finely ground $(NH_4)_2HPO_4$ were added to the yeast extract and the mixture shaken until the salt was completely dissolved. Concentrated ammonia was then slowly added at the rate of 30 ml. for each 100 ml. of extract; during the addition the extract was vigorously shaken. The extract was left overnight in the refrigerator. The following day the extract was filtered through a double filter using suction. The filtrate was placed in large flat porcelain trays in a cold draught tunnel and left until the volume was reduced to about 350 ml. During this evaporation all the free ammonia and the alcohol were driven off. The whole of the yeast extract concentrate prepared in this way was incorporated in each 2 kg. of diet.

Purification of liver extract. 120 g. of liver extract (Boots) and 7·2 g. $(NH_4)_2HPO_4$ were dissolved in distilled water and roughly neutralized with NaOH. The volume was adjusted to 480 ml. and 120 ml. NH_4OH (sp. gr. 0·88) were added with constant stirring. The liquid was left overnight in the refrigerator and filtered by suction the following day. The filtrate was exposed on flat porcelain trays in the cold draught tunnel until all the ammonia had been driven off. The extract was then collected, the trays were rinsed with distilled water and the whole made up to 400 ml.

Preparation of inorganic constituents. KCl and KH_2PO_4 were purified by crystallization from boiling saturated aqueous solutions made slightly acid with HCl. NaCl was purified by precipitation from saturated aqueous solution by HCl. $NaHCO_3$ was purified by precipitation from warm saturated aqueous solution by C_2H_5OH. $CaCO_3$ was prepared by double precipitation from dilute $CaCl_2$ solution by $(NH_4)_2CO_3$. It was found that these salts, even when purchased in the most highly purified forms, contained sufficient Mg to increase markedly the Mg content of the diet.

Purification of casein. 1 kg. of casein in small portions at a time was ground with 0·2 % acetic acid until the whole was suspended in a volume of 10 l. in a 12 l. bottle. The bottle was shaken from time to time and the following day the casein was collected by filtering through a linen bag. The last portions of the washing liquor were removed in a hand press. The second washing was with distilled water, the casein being as before ground down in a mortar. The washings were carried out for 10 days, 5 acetic acid and 5 distilled water washings in all being used alternately. The casein was then suspended in 5 l. of 70 % C_2H_5OH for 24 hr. and, after filtering, in the same volume of 90 % C_2H_5OH for a similar period. Finally the casein was dried in the cold draught tunnel and then in an electric oven at 65°.

Purification of starch. Starch, as commercially obtained, frequently contains a large amount of grit. This was removed by suspending 500 g. of starch in

[1] We wish to acknowledge our indebtedness to Dr R. L. Mitchell, of the Macaulay Institute, who performed this analysis.

2 l. of 10 % HCl in a tall cylinder and allowing to stand for 5 min. The suspension was then siphoned off to within 3 in. of the bottom of the cylinder, the remainder, which contained most of the grit, being discarded. The starch was left for 24 hr. in the acid solution with occasional shaking, filtered by suction and washed with large volumes of distilled water. It was then suspended twice more in acid and twice washed on the funnel. After the third day's washing it was dried in the cold draught tunnel and finally in the oven at 65°. The sample was then pulverized.

Paper pulp. As a source of roughage paper pulp was added to the diet. Crude compressed pulp was ground in a mill, suspended in 2·5 % HCl for 24 hr. and then washed by suction with distilled water. This washing was repeated twice and the pulp finally dried.

Butterfat. Butter from a constant source was purified as required. Freshly made butter was melted and filtered through glass wool to remove curd and then through filter paper. The filtered fat was shaken in a separating funnel with an equal volume of hot 2 % HCl and the aqueous layer discarded. The washing was repeated twice. The fat was then washed 3 times with equal volumes of hot distilled water. The complete diet, with the exception of the sources of vitamin B complex, is given in Table 1.

Table 1. *Composition of diet*

Casein	400 g.
Starch	1020 g.
Butterfat	180 ml.
$CaCO_3$	30 g.
KCl	20 g.
$NaHCO_3$	14 g.
KH_2PO_4	28 g.
NaCl	6 g.
$MnSO_4, 4H_2O$	0·2 g.
$CuSO_4, 5H_2O$	0·2 g.
KI	0·02 g.
Radiostoleum	2 ml.

Preparation of the diet. The starch, casein and inorganic salts (except those of the three trace elements) were thoroughly mixed. The radiostoleum was dissolved in the melted butterfat and the fat poured into the dry materials; the whole was well mixed until all the fat was blended into the mixture. To this was then added the yeast extract to which had been added the trace elements dissolved in a few ml. of water. After complete mixing the diet was weighed, placed in the cold draught tunnel until the weight was reduced to 2 kg. and then stored in the refrigerator.

The daily ration was prepared by weighing 5 g. of paper pulp and adding to this 10 ml. of the liver extract, 1 ml. of wheat germ oil and a sufficient amount of distilled water so that when 100 g. of the dry diet were added the whole formed a stiff dough. In cases where a Mg-adequate diet was needed 10 ml. of a 7 % $MgSO_4, 7H_2O$ solution were also added.

Lister hooded rats were used in Exp. 1 and albino rats in Exps. 2–4. All rats were weaned at 25 days of age and transferred to their experimental diets, only those of good weight and appearance being used.

All animals were housed in galvanized iron cages with screen bottoms and no bedding was used. Rats on the deficient diet were housed individually after the 4th day on the diet to avoid cannibalism which otherwise had been found to occur. To avoid precipitating seizures in rats on deficient diets, all the rats were kept in an almost sound-proof, constant temperature (25°) room. Food and water were offered *ad lib.* All animals, other than those dying in tetany, were killed by coal gas.

Analytical methods

After slaughter the skeleton was isolated quantitatively according to the method of Subrahmanyan *et al.* [1939]. All analyses were performed on a solution of the bone ash in HCl.

Calcium. The estimation was carried out by a slight modification of the method of Clark & Collip [1925], the supernatant washing liquors being siphoned off to prevent the loss of floating particles of Ca oxalate.

Magnesium. The estimation was performed according to the method of Godden & Duckworth [1935].

Phosphorus. The estimation was performed by the method of Fiske & Subbarow [1925].

Exp. 1. Comparison between the synthetic diet supplemented with Mg and the stock diet of the colony

Before proceeding with the actual trials it was necessary to ensure that the diet, as prepared above, when supplemented with nothing but a pure Mg salt, should be adequate for normal growth and reproduction. Proof of this was obtained in the following experiment. Six pairs of female litter-mates were divided into two groups, one of each pair being allotted to each group. Group 1 received the Mg-deficient diet supplemented with Mg (as its sulphate) and glass-distilled water. Group 2 received the stock diet of the Institute colony (details of which have been given by Thomson [1936]) and skimmed milk, tap-water and greenstuff *ad lib.*

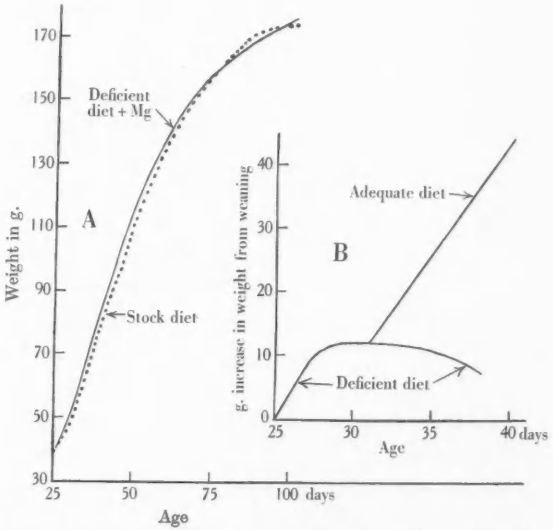


Fig. 1. A. Comparison of growth rates of two groups of rats, one receiving the deficient diet supplemented with Mg and the other receiving the stock diet of the main colony. B. Growth rates of rats receiving the Mg-deficient diet until death occurred and of rats given the Mg-adequate diet after 6 days on the deficient diet.

The growth data for these two groups of rats are given in Fig. 1 A. It will be clearly seen that the rats on the synthetic diet which had been supplemented

with nothing but pure Mg sulphate grew normally and had as good a rate of growth as those on the stock diet.

Breeding tests were carried out on the two groups of females, starting at 105 days of age and using the same stock males. Whilst the group on the stock diet bred normally, the does on the synthetic diet plus Mg but without the wheat germ oil did not breed until tocopherol acetate was administered for 10 days. These results indicate that the diet, as shown in Table 1 but without the wheat germ oil, was entirely adequate for normal growth when supplemented with pure $MgSO_4$ but was deficient in vitamin E. For this latter reason wheat germ oil, in the amount shown, was incorporated in the diet for the remaining experiments.

Exp. 2. The effects of Mg deficiency on the growth and calcification of the skeleton and on the composition of the bone ash

Seven pairs of female and three pairs of male litter-mates of similar weaning weights were allotted, one of each pair to the deficient diet group and the other to the adequate diet group. Each pair continued on experiment until the rat on the deficient diet died, when the corresponding litter-mate on the adequate diet was killed. Such deaths as occurred under observation were typical of Mg deficiency; animals found dead had, as judged by the position and posture of the cadaver, died similarly.

After death the left femur, tibia, fibula, radius, ulna, humerus and scapula were dissected free from adhering tissue and analysed separately. The remainder of the skeleton was cleaned according to the technique recorded above. The separate analysis of the left leg bones was carried out to obtain data for the amount of organic matter in the skeletons; these cannot be obtained from trypsin-cleaned skeletons owing to partial hydrolysis of the organic matrix by the trypsin.

In this and the two subsequent experiments it was found that in all cases the rats on the synthetic diet deficient in Mg failed to grow normally and after about 4 days there was cessation of growth and, in animals with longer survival periods, a tendency to lose weight. Restitution of Mg to the diet after 6 days on the deficient diet produced almost normal rates of growth. These are illustrated in Fig. 1 B where a curve showing the average gains from weaning weight during the period on the experimental diets is given. This curve has been constructed from the combined data of Exps. 2–4.

There was less growth of skeleton in deficient animals which survived for longer periods on the diet than in their litter-mate controls. Owing to the normal variation between litter-mates the small differences between animals with short survival periods cannot be regarded as significant. In all cases there was a poorer degree of calcification of the organic matrix in the skeletons of rats fed on the deficient diet than in controls and this difference was statistically significant. The reduction in calcification bore no relation to survival period.

The amount of Mg in the skeletons of rats fed on the deficient diet was in all cases less than that of litter-mate controls. In the two animals which survived the longest (D 9 and D 10) the values were minimal (see Table 2).

The percentage of Ca in the skeletal ash was, with one exception, always somewhat higher in the deficient rat than in its litter-mate control; the mean difference was statistically significant. There was no significant difference in the percentage of P in the skeletal ash of the two groups. The percentage of Mg in the ash was lower with increasing survival on the deficient diet and was generally about half that of the corresponding control animal (see Table 2).

Table 2. *Exp. 2. Ca, P and Mg contents of the total skeletal ash and the percentage ash in the leg bones of rats receiving diets deficient or adequate in Mg*

Rat no.	Days on diet	Wt. skeletal ash mg.	Total mg. in ash of			% in ash of			Leg bones: Wt., dry fat-free mg.	Leg bones: % ash
			Ca	P	Mg	Ca	P	Mg		
D 1	6	1608	609	293	9·2	37·8	18·2	0·57	371	53·5
D 2	9	1622	618	290	8·3	38·1	17·9	0·51	343	52·6
D 3	9	1630	631	293	11·9	38·7	18·0	0·73	379	53·0
D 4	9	1761	699	320	9·1	39·7	18·2	0·52	407	52·7
D 5	9	1836	681	324	9·1	37·0	17·7	0·50	436	53·0
D 6	10	1684	661	307	8·4	39·3	18·2	0·50	379	54·2
D 7	10	1846	718	336	9·0	38·9	18·2	0·49	415	56·9
D 8	11	1900	731	338	9·4	38·5	17·8	0·49	416	56·9
D 9	16	1615	635	296	6·4	39·3	18·3	0·39	346	54·3
D 10	23	1915	756	341	6·6	39·5	17·8	0·34	452	55·2
					Average	38·7	18·0			54·2
					S.D.	±0·8	±0·2			±1·5
A 1	6	1552	585	288	14·1	37·6	18·6	0·91	322	56·7
A 2	9	1585	592	287	16·1	37·3	18·1	1·01	344	56·4
A 3	9	1784	686	320	15·2	38·4	17·9	0·85	399	55·8
A 4	9	1979	744	354	18·2	37·6	17·9	0·92	428	57·4
A 5	9	2041	765	369	18·4	37·5	18·1	0·90	429	56·6
A 6	10	1946	751	356	17·1	38·6	18·3	0·88	411	57·3
A 7	10	2007	776	361	17·8	38·7	18·0	0·89	348	57·6
A 8	11	2045	771	376	18·8	37·7	18·4	0·98	416	57·1
A 9	16	2579	978	471	21·3	37·9	18·3	0·83	497	59·6
A 10	23	3261	1246	582	24·3	38·2	17·8	0·74	756	57·9
					Average	38·0	18·1			57·2
					S.D.	±0·5	±0·2			±1·0

Rats on deficient diet numbered D 1 to D 10; corresponding litter-mates on adequate diet numbered A 1 to A 10.

Exp. 3. The effect of Mg deficiency and subsequent restitution of Mg in the diet on the composition of femora of rats

Five litter-mates of both sexes and of closely similar body weight were selected from each of 8 litters. The five chosen from each litter were allotted one to each of the groups described below, the distribution being arranged so that each group contained 4 male and 4 female rats. The groups were treated as follows:

Group 1. Rats were killed at the time of weaning (25 days).

Group 2. Rats received the deficient diet for 6 days and were then killed.

Group 3. Rats received the adequate diet for 6 days and were then killed.

Group 4. Rats received the deficient diet for 6 days after which they received the adequate diet for 10 days; they were then killed.

Group 5. Rats received the adequate diet for 16 days and were then killed.

At death the femora were dissected free from all adhering flesh. After alcohol and ether extraction the eight femora from each group were bulked and the analyses were carried out. The results are shown in Table 3.

There was very little difference between the dry fat-free weight of the bulked femora of the 6-day deficient and of the 6-day adequate groups. Unexpectedly, a greater difference appeared between Group 4 (6-days deficient followed by 10-days adequate) and Group 5 (16-days adequate). As was found in Exp. 2, there was a lower percentage of calcification in the deficient animals than in the adequate controls. Since the weights of dry fat-free bone were the same this

Table 3. *Exp. 3. The ash and Ca, Mg and P contents of the bulked femora of groups of rats receiving diets deficient and/or adequate in Mg*

Group	Wt., dry fat-free mg.	Wt. of ash mg.	% of ash	Total mg. in ash of			% in ash of		
				Ca	P	Mg.	Ca	P	Mg
1. Weaning controls	536	264	49·2	97·2	48·4	2·82	36·8	18·3	1·07
2. 6 days deficient	822	415	50·5	161·4	74·1	1·95	38·9	17·8	0·47
3. 6 days adequate	826	433	51·8	165·6	79·0	3·79	38·2	18·2	0·88
4. 6 days deficient and 10 days adequate	1290	713	55·2	279·5	129·8	5·76	39·2	18·2	0·81
5. 16 days adequate	1356	755	55·7	290·6	135·0	6·52	38·5	17·9	0·86

indicated an increased growth of organic matrix during the first 6 days. The difference in the percentages of ash was somewhat reduced after 10 days on the adequate diet after the deficiency period, as shown by the data for Groups 4 and 5, thus indicating an improvement in the degree of calcification of the matrix.

Group 2 showed deposition of Ca and P when compared with the weaning value, Group 1. The Mg values indicated that during the 6-day deficiency period there was marked mobilization of Mg (0·87 mg.) and the difference between Groups 2 and 3 was 1·84 mg. During the 10-day period of adequacy, the amount of Mg deposited in Group 5 was 2·73 mg. more than that in Group 3. Group 4, during a similar period, deposited 3·81 mg. more than its parallel control Group 2, although the deposition of organic matrix and bone salt was less than in the case of Group 5. Group 1 showed a Ca : Mg ratio of 34·5, Group 2 of 82·8, Group 3 of 43·7, Group 4 of 48·5 and Group 5 of 44·6. The large reduction in total bone Mg in Group 2 was not made up in 10 days on the supplemented diet, as indicated by the results for Group 4. The Ca : Mg ratio of the amounts of Ca and Mg deposited during the last 10 of the 16 days on the adequate diet (i.e. Group 5 values minus Group 3 values) was 45·8. On the basis of this same ratio it may be calculated from the amount of Ca deposited by the animals of Group 4 that they should have deposited 2·58 mg. Mg during the 10 days of adequate diet, and this amount added to the value 1·95 for the amount of Mg in Group 2 gives the figure 4·53 mg. Mg for the total amount which might be expected at the end of 16 days in Group 4 animals. This is 1·23 mg. less than the amount actually found in Group 4, this 1·23 mg. representing the extra Mg deposited by Group 4 over and above that deposited by the controls. It is realized that such a calculation is open to the criticism that the Group 4 bone development lagged slightly behind the Group 5 development but the difference cannot vitiate the conclusion that the changes were indicative of the replenishment of a reserve. This is discussed below.

Exp. 4. The effect of small variations in the Mg content of Mg-deficient diets on the composition of skeletal ash

Four female rats from each of 6 litters were divided among 6 groups. The division was such that one rat from each litter was allotted to Group 1 and another to Group 6. The remaining two rats were placed one in each of 2 of the 4 intermediary groups. The groups were as follows:

Group 1.	Deficient diet		(Total Mg, 6 p.p.m.)
Group 2.	,,	+ 3 p.p.m. Mg	(Total Mg, 9 p.p.m.)
Group 3.	,,	+ 6 p.p.m. Mg	(Total Mg, 12 p.p.m.)
Group 4.	,,	+ 9 p.p.m. Mg	(Total Mg, 15 p.p.m.)
Group 5.	,,	+ 12 p.p.m. Mg	(Total Mg, 18 p.p.m.)
Group 6.	,,	+700 p.p.m. Mg	

Group 6, by this addition of Mg, became the adequate diet as in all the preceding experiments.

All rats were killed after 6 days on the diets. All the rats of each litter were placed together and subjected to an auditory stimulus to precipitate a convulsive seizure except such animals as spontaneously reacted to handling alone. The results of the stimulation are included in Table IV.

Table 4. *Exp. 4. Sketetal ash and Ca, P and Mg of the ash of rats receiving diets adequate and deficient in Mg*

Rat no.	Mg in 100 g. diet mg.	Skeletal ash mg.	Total mg. in ash of			% in ash of		
			Ca	Mg	P	Ca	Mg	P
1–L 1*	0·6	1499	580	9·0	281	39·0	0·60	18·9
1–L 2†	0·6	1297	493	7·5	242	38·0	0·58	18·6
1–L 3	0·6	1298	493	7·5	247	38·0	0·58	19·0
1–L 4*	0·6	1395	530	8·7	248	38·0	0·62	17·8
1–L 5*	0·6	1406	530	8·2	256	37·7	0·59	18·2
1–L 6†	0·6	1151	434	6·2	201	37·7	0·54	17·4
2–L 1†	0·9	1615	623	9·3	296	38·6	0·58	18·3
2–L 2*	0·9	1334	509	7·8	250	38·2	0·58	18·7
2–L 3†	0·9	1348	509	8·2	256	37·8	0·60	19·0
3–L 1*	1·2	1439	561	8·6	269	39·0	0·60	18·7
2–L 4	1·2	1383	530	8·2	247	38·3	0·60	17·9
2–L 5	1·2	1532	581	8·9	274	37·9	0·58	17·9
3–L 2†	1·5	1320	501	7·8	252	38·0	0·59	19·1
3–L 4†	1·5	1437	543	7·6	256	37·8	0·53	17·8
2–L 6*	1·5	1540	589	8·2	267	38·2	0·53	17·3
3–L 3	1·8	1360	509	7·9	254	37·4	0·58	18·7
3–L 5	1·8	1431	543	7·8	253	37·9	0·54	17·7
3–L 6*	1·8	1473	556	7·8	256	37·7	0·53	17·4
4–L 1	70·0	1594	604	15·0	301	37·9	0·94	18·9
4–L 2	70·0	1485	552	15·2	279	37·2	1·02	18·8
4–L 3	70·0	1331	497	13·2	252	37·3	0·99	18·9
4–L 4	70·0	1537	573	13·3	280	37·3	0·86	18·2
4–L 5	70·0	1565	585	13·5	282	37·4	0·86	18·0
4–L 6	70·0	1561	581	13·8	274	37·2	0·88	17·6

In the serial numbers the first digit refers to the numbers of the rat within the litter and the second digit to the number of the litter.

* Seizure induced by auditory stimulus.

† Seizure occurred spontaneously.

Those not marked did not react to prolonged stimulus.

Spontaneous seizures were not restricted to animals on the lowest Mg intake (6 p.p.m.), in fact one animal in this group (1–L 3) did not react to prolonged stimulation. Again, while no spontaneous seizures occurred in the groups receiving 12 and 18 p.p.m. (and only in one case in each group could seizures be induced by auditory stimulus), the intermediary group, receiving 15 p.p.m. Mg, contained two cases of spontaneous and one case of induced seizures. This variability in response suggests that the individual susceptibility of the animal overshadows the effect of variations in Mg intake at low levels.

The data in this experiment confirm those of previous ones. Although Groups 2–5 contained too few rats for a comparison of the averages with that of Group 6, it was noted that deficient rats in any one litter had higher percentages of Ca in the bone ash than the corresponding litter-mates fed on the adequate diet.

The point of greatest interest is, of course, the amount of skeletal Mg. Inspection shows that the rats in Groups 1–5 inclusive had about half the

skeletal Mg of rats in Group 6. Although Group 5 received 3 times the Mg intake of Group 1 the total skeletal Mg values and the percentages of Mg were about the same. Differences of the order used in this experiment were without effect on skeletal Mg.

Discussion

Orent *et al.* [1934] reported experiments on bone growth in rats receiving a diet containing only 0·00018 % Mg (1·8 p.p.m.). They stated that there was a marked acceleration in bone growth caused mainly by excessive deposition of Ca. This acceleration was found to occur during the first 5 days on the diet; the subsequent course of bone growth did not differ from that of the control animals although the initial difference was maintained. It was also found that in spite of the low intake of Mg the bone Mg increased during the experimental period, although at a rate less than that of the control animals.

It is felt that this accumulation of Ca in the bone is remarkable since the increase in the percentage of P was not proportional and large depositions of Ca without a corresponding increase in anion are difficult to understand unless a fundamental change in the composition of bone is postulated. Further, the accumulation of Mg in the skeleton during a period when practically no Mg was contributed by the diet seems to us biologically unlikely since it involves a gain in Mg by a principally structural organ at the expense of functional organs. This is even more remarkable when it is considered that these authors had previously reported [Kruse *et al.* 1932] that their animals gained weight on the deficient diet; the amount of Mg available per unit weight of tissue would thus have been even further reduced. Also, this conception that Mg moves to the skeleton in spite of almost complete absence of Mg from the diet and in the face of increasing weight of the soft tissues leads to the conclusion that the Mg of the soft tissues has at least the partial function of a reserve upon which the skeleton could draw to remedy transitory insufficiencies. Such a view seems improbable.

Finally, the data presented are open to certain criticisms. The bone ash analyses show erratic and, in some cases, extremely low Ca : P ratios. The ratio, for example, of the control animals at weaning was given as 1·01 whereas normal animals generally approximate to 2·00. A further criticism is that the sum of the oxides of Ca, Mg and P ($CaO + MgO + P_2O_5$) generally accounts for less of the ash than is reported by other workers, the oxides accounting for only 83 % of the ash in the case of the weaning control animals instead of the usually accepted amount of about 96 % [e.g. Hammett, 1925, 1, 2, 3]. Also, the values given for the Mg content of the bone ash of weaning controls (which must be considered as basic to any investigation of reserve mobilization) were only two-thirds of those recorded by Hammett. Our data are in agreement with those of Hammett.

In the present experiments the symptomatology of the deficiency was similar to that reported by other workers on Mg deficiency. Peripheral vasodilatation was observed early in the experiment, its maximum severity being reached by the 4th day and thereafter gradually diminishing until it had completely disappeared by the end of the 5th day. Kruse *et al.* [1932] found that the first appearance of vasodilatation occurred between the 3rd and 5th days and increased in intensity until the 12th day, after which it gradually disappeared. These authors also found that the 11th day was the earliest upon which convulsions occurred, the average duration of deficiency before the first seizure being 18 days. In our case we found that convulsions either occurred

spontaneously or, generally, could be induced from the 6th day onwards. Very few animals survived beyond 12–15 days although occasional subjects lived beyond 20 days. The additional symptoms observed in cases with longer survival periods were very similar to those reported by Tufts & Greenberg [1937–8, 1], namely, ringed-eye dermatitis, roughness of the coat and, in one case surviving 23 days, oedema. In general appearance and response to restitution of Mg in the diet, our rats were identical with those photographically recorded by these workers.

Since the rats in the present investigation had a much shorter survival period than those in the researches of Kruse *et al.* [1932], and of Tufts & Greenberg [1937–8, 1] the explanation which might immediately suggest itself is that there was an additional deficiency to that of Mg. This, we feel, is unlikely because (1) rats fed on the diet supplemented with Mg showed normal growth rates, and (2) the symptomatology did not differ from that found by other workers. The explanation for the difference between a complete Mg deficiency syndrome requiring 6–9 days and one requiring 4–5 weeks must be sought elsewhere and this, we suggest, is in the Ca : P ratio of our diet as compared with that used by other workers, together with the low level of Mg intake which we attained.

The outstanding metabolic change in animals on Mg-deficient diets is the disturbance in Ca metabolism; this has been reported both in rats [Tufts & Greenberg, 1937–8, 1, 2] and in calves [Moore *et al.* 1938]. There is an abnormal deposition of Ca in the soft tissues and, consequently, any factor which would accelerate or intensify disturbances in Ca metabolism might reasonably be expected to increase either the severity or rapidity of onset of the syndrome of Mg deficiency. Thus, Tufts & Greenberg [1937–8, 1, 2] found convulsions occurring earlier on a diet containing 1·19 % Ca, 0·60 % P and 0·005 % Mg than on a diet containing 0·88 % Ca, 0·68 % P and only 0·002 % Mg.[1] Throughout their experiments these authors found that an increase in the Ca intake increased the requirements of Mg for survival and normal development. In our case the intakes were 0·60 % Ca, 0·30 % P and only 0·0006 % Mg. On account of the above observations together with the recognized influence of the ratio Ca : P upon the metabolism of these two elements we feel that the contraction of the syndrome period which we found is best interpreted as an effect of a higher Ca : P ratio together with a low Mg intake.

The effect of Mg deficiency on the growth of the skeleton was slight during the first 6 days of deficiency. There was, however, a reduction in the ratio of inorganic matter to organic matrix during this period (Exp. 3) but the condition did not become progressively worse with longer periods on the deficient diet (Exp. 2). Judging from the total skeletal ash and from the reduction which occurred in food consumption this cessation of growth and maintenance of the slightly imperfect degree of calcification might have been caused by inanition. The animals with longer survival periods had lower amounts of skeletal ash than their litter-mates on the adequate diet. This observation is contrary to that of Orent *et al.* [1934] who found that Mg deficiency increased bone growth and also increased the percentage of ash in bone.

Mean values for the percentage of Ca in skeletal ash showed that throughout all experiments the Mg-deficient animals had higher values than adequately fed

[1] Tufts & Greenberg do not give a complete analysis of their diet. The above values are calculated from the composition of the salt mixture used. The amounts of Ca and P contributed by their other dietary constituents would not, however, be sufficient seriously to affect the above values.

litter-mates. In only one individual case was this otherwise. The mean differences were slight (0·5–0·7 %) but significant. Since this occurred concomitantly with a reduction in the amount of Mg in the ash it might be suggested that a replacement of mobilized Mg by Ca took place, especially since the amounts were closely similar when compared on the basis of equivalents. While this may be correct, and, in view of the studies of Gruner *et al.* [1937] it seems probable, it cannot be regarded as completely established by the data since the differences involved were close to the analytical error of the method in the case of Ca. Also, restitution of Mg in the diet in Exp. 3 did not abolish the difference in the percentages of Ca in the bone ash; but again the difference lay close to the analytical error of the method used. Incidentally the increase in the percentage of Ca was only one-quarter of that reported by Orent *et al.* [1934].

About one-third of the skeletal Mg was lost from the skeleton during the first 6 days on the deficient diet. Among the animals surviving 11 days or less on the deficient diet (Exp. 2) the differences in the total skeletal Mg bore no relation to the length of the survival period. There was a tendency for gradual reduction in the percentage of Mg in the skeletal ash, but such a reduction cannot be interpreted as evidence of slight continued mobilization since it could, and probably did, arise through small depositions of Mg-low, or even Mg-free, bone salt. The animals with the longest survival periods (D 9, 16 days; D 10, 23 days) showed the lowest total skeletal Mg, both values being well below those of animals dying earlier on the diet. From these data, together with the data for the first 6 days in Exp. 3, the conclusion can be drawn that about one-third of the skeletal Mg can be easily mobilized; whether the remainder can be mobilized in periods of longer survival is not yet clear. Unless it is argued that cessation of growth reduced the demand of the soft tissues for Mg after the first 6 days on the deficient diet, the conclusion can be drawn that a difference in the availability of the two fractions of skeletal Mg points to a difference in the forms in which they occur in bone.

That a portion at least of bone Mg functions as a reserve is illustrated by the results of Exp. 3. Restitution of Mg to the diet resulted in a deposition of Mg in relation to Ca considerably beyond that required for the formation of normal bone salt. The increment deposited above that required for the newly formed bone salt appears therefore to have fulfilled the role of replenishment of a partially exhausted reserve.

It will be seen from the above that our results are contrary to those of Orent *et al.* [1934]. Their observation that the skeleton not only does not act as a reserve for Mg but that actual deposition of Mg occurs in acute Mg deficiency is not supported. Our present view is, to some extent, supported by the work of Tufts & Greenberg [1937–8, 1, 2]. These workers found that the Mg of soft tissues was only slightly reduced in acute Mg deficiency, that total body Mg was usually not reduced and that a fairly good body growth was maintained up to the time of hyperirritability. Their data do not permit of recalculation, but the inference from their observations is that the Mg of the soft tissues, under conditions of constant total body Mg, must have been maintained at the expense of the skeleton. These and our own results do not support the view of Orent *et al.* [1934] that "bone has a stronger attractive force than any other tissue for Mg".

A further observation of Orent *et al.* [1934] was that skeletal Mg was, in part, mobilized during convulsions and that this movement was sufficiently rapid to be completed within 2 min. Exp. 4 offered an opportunity to confirm the observation. We were unable to find any difference between the Mg content of the skeletons of animals dying in convulsions, spontaneous or induced, and that

of animals in which convulsions could not be induced. In order to avoid as far as possible any effect being produced by coal gas the animals which did not develop convulsions were gassed and decapitated as soon as they were unconscious. The mean amount of Mg mobilized was about 0·04 mg. for the hind legs alone and this is equivalent to a mobilization for the whole skeleton of about 3 mg. Mg. The increases which these workers found in blood Mg at the time of seizure were slight and, even if the interstitial fluid is taken into account as well, the changes only account for a very small fraction of the bone Mg said to have been mobilized.

The variability in the response to auditory stimuli on the 6th day of deficiency on the part of the deficient rats in Exp. 4 suggests that the incidence of tetany in Mg deficiency is not referable simply to disturbance in body fluid Mg. The differences observed ranged from spontaneous fatal seizure to complete failure to react to prolonged stimulation. Greenberg & Tufts [1938] found that injection of Mg several hours before stimulation did not prevent seizures; they showed in further studies that a mid-brain lesion was involved. Our results agree better with such a view than with the conception that the tetany of Mg deficiency is the outcome of disturbances in tissue fluid composition arising from the deficiency.

In the present experiments it has been noted that rats surviving for longer periods have more fragile bones than normal rats of the same age or, in fact, of much younger rats which have been reared on adequate rations. In this observation we confirm Lavollay [1936], who observed the same increase in fragility under conditions of Mg deficiency. He was, however, unable to find any evidence of abnormality on roentgenographic examination. In addition to the fragility we noticed that the ashes of bones presented an unusual appearance in that there was extensive "pitting". Since the degree of calcification of the organic portion of the bone is only slightly affected in Mg deficiency it seems probable that the fragility arises from other causes. In preliminary studies of the histology of the bones we have obtained some evidence of disordered growth of the organic matrix of the shaft of the leg bones and it seems possible that this may explain the increased fragility observed.

Summary

A method of preparing a Mg-deficient diet is described.

It has been shown that the skeleton can, under conditions of acute Mg deficiency at least, function as a reserve of this element upon which the rat can make large drafts. This effect is reversible as shown by experiments in which Mg was restored to the diet.

REFERENCES

Clark & Collip (1925). *J. biol. Chem.* **63**, 461.
Duckworth (1938–9). *Nutr. Abstr. Rev.* **8**, 841.
Fiske & Subbarow (1925). *J. biol. Chem.* **66**, 375.
Godden & Duckworth (1935). *Biochem. J.* **29**, 445.
Greenberg & Tufts (1938). *Amer. J. Physiol.* **121**, 416.
Gruner, McConnell & Armstrong (1937). *J. biol. Chem.* **121**, 771.
Hammett (1925, 1). *J. biol. Chem.* **64**, 409.
—— (1925, 2). *J. biol. Chem.* **64**, 685.
—— (1925, 3). *J. biol. Chem.* **64**, 693.
Kruse, Orent & McCollum (1932). *J. biol. Chem.* **96**, 519.
Lavollay (1936). *Actualités Scientifiques et Industrielles*, no. 420.
Moore, Hallman & Sholl (1938). *Arch. Path. Lab. Med.* **26**, 820.
Orent, Kruse & McCollum (1934). *J. biol. Chem.* **106**, 573.
Subrahmanyan, Duckworth & Godden (1939). *Biochem. J.* **33**, 1421.
Thomson (1936). *J. Hyg., Camb.*, **36**, 24.
Tufts & Greenberg (1937–8, 1). *J. biol. Chem.* **122**, 693.
—— —— (1937–8, 2). *J. biol. Chem.* **122**, 715.

18. A NOTE ON THE POSSIBLE RELATIONSHIP OF THE CARBOHYDRATE COMPONENT OF PROTEINS TO THEIR ANTIGENIC PROPERTIES

BY A. NEUBERGER[1] AND M. E. YUILL

From the Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

(*Received 29 November 1939*)

THE classical early studies of Avery & Heidelberger [1925] on pneumococcal antigens, followed by numerous investigations on other organisms by various workers, have shown that bacterial antigens in general owe their immunological properties not to their protein constituents alone but to protein-carbohydrate complexes, in which the carbohydrate component exercises a determinant influence with respect to immunological specificity. It is now generally recognized that most proteins contain carbohydrate groups as integral parts of their molecules, and the question therefore arises whether such carbohydrate groups may be of importance in determining the specificity of the immunological reactions to which they give rise.

It can scarcely be maintained that the presence of a carbohydrate group is indispensable to the development of antigenic activity, since there exist proteins, such as haemoglobin and pepsin, which contain no carbohydrate but nevertheless act as antigens. The possibility remains however that those proteins which do contain carbohydrate owe their immunological specificity, in part at least, to the presence of this constituent.

It has indeed already been claimed by Bierry [1931; 1934] that chemical differences between the carbohydrate groups of the serum proteins of different species are responsible for the species specificity of these proteins; no serological evidence however was offered in support of this conclusion. All attempts to demonstrate actual serological reactions between polysaccharides isolated from a protein and the antiserum against the same protein have in fact so far been unsuccessful [cf. e.g. Ferry & Levy, 1934], although Sevag & Seastone [1934] isolated a substance from egg white, containing 11·65% N and giving positive carbohydrate tests, which produced anaphylactic shock in guinea-pigs sensitized with egg white; this substance however was not chemically characterized, and in view of its high nitrogen content the presence of protein or peptide impurities cannot be excluded. The whole of the previous work on this subject is indeed open to the criticism that the so-called polysaccharides which have been employed have not been rigidly proved either to be free from protein or to be themselves other than artefacts produced in the process of their isolation.

One of us [Neuberger, 1938] has recently described the isolation of a polysaccharide from egg albumin which can reasonably be regarded as chemically unaltered and as representing an integral constituent of the original protein molecule; the possession of this and of a similar product derived from ovomucoid has made it possible to subject the hypothesis of the connexion between the carbohydrate groups and immunological properties of proteins to rigid experimental test.

[1] Beit Memorial Research Fellow.

Experimental

The egg albumin used for these experiments was a sample which had been recrystallized 5 times. The ovomucoid was prepared by heating egg white to 80° for 5 min., filtering off the denatured albumin and precipitating the solution with 5 vol. alcohol; the precipitate was again dissolved in water and heated to 80°, a small amount of insoluble material being filtered off, and the protein was precipitated from the filtrate with 3·5 vol. alcohol; the process of solution and precipitation was repeated once more. The polysaccharides were prepared according to Neuberger [1938], the method being slightly modified in the case of ovomucoid.

For each series of experiments groups of 3–6 rabbits were used. The animals immunized against egg albumin were given five courses of injections each consisting of 9 doses administered over a period of 3 weeks; the doses were increased in succeeding courses from an initial dose of 10 mg. in the first course to a final dose of 70 mg. in the fifth course. The rabbits immunized against ovomucoid were similarly treated except that they only had four courses of injections. In all cases an interval of 2–3 weeks was allowed between the successive courses.

At the end of the period of immunization ring precipitation tests were performed in the usual manner; bulk precipitation tests were carried out by mixing equal volumes of different dilutions of serum with varying dilutions of antigen. For complement fixation tests there were generally used 2 M.H.D. of complement and 2–3 M.H.D. of haemolysin, 1–2 hr. at 37° being allowed for fixation of the complement by the antigen-antibody mixture.

Results

Precipitation reactions carried out by the ring technique between the anti-egg albumin sera and egg albumin were strongly positive up to dilutions of 1 : 100,000. In the earlier series of experiments a faintly positive reaction was obtained between the anti-egg albumin sera and the egg albumin polysaccharide; this positive ring test only occurred however at high concentrations (antigen dilutions of 1 : 200–1 : 500) and in later experiments it was not observed at all. Attempts to inhibit the reaction between egg albumin and its antiserum by first incubating the latter with different dilutions of the polysaccharide were completely unsuccessful, as were also efforts to demonstrate bulk precipitation between the polysaccharide and the anti-egg albumin serum.

After the first course of injections complement fixation tests were uniformly negative; at the end of the third course slight fixation of complement was observed with all three sera at a dilution of 1 : 200 of the polysaccharide; this reaction however failed to persist in one out of the three sera after the fifth course of injections. In an earlier series of injections one out of three sera gave optimal fixation of complement with 50 % haemolysis at a dilution of 1 : 3200 of the polysaccharide.

In spite of the fact that ovomucoid has hitherto been regarded as a poor antigen [Lewis & Wells, 1927] the antisera which we obtained after five courses of injections reacted with the ovomucoid used as antigen to a dilution of 1 : 100,000. All attempts however to demonstrate direct reaction between the ovomucoid antisera and the polysaccharide derived from ovomucoid either by the ring or bulk precipitation tests or by complement fixation were unsuccessful, nor was it possible to demonstrate inhibition of the reaction between ovomucoid and its antiserum by means of the polysaccharide.

The results which we have obtained with the two proteins which we have investigated do not therefore afford any clear evidence that the polysaccharide component plays a part in the immunological reaction; the few positive results which were obtained with egg albumin were very feeble and moreover were not entirely reproducible, whilst our results make it clear that the polysaccharide component of ovomucoid is of no significance in the immunological reactions of the protein, in spite of the fact that the content of carbohydrate in ovomucoid is very high. It seems to us therefore to be unlikely in general that the immunological properties of proteins are significantly affected by the carbohydrate groups which the latter may contain.

Summary

The possible importance of the carbohydrate groups of egg albumin and ovomucoid in the serological reactions of these proteins has been studied by specific precipitation (ring and bulk techniques), complement fixation and serological inhibition reactions; no evidence has been obtained that the carbohydrate groups play any significant part.

REFERENCES

Avery & Heidelberger (1925). *J. exp. Med.* **42**, 372.
Bierry (1931). *C.R. Acad. Sci., Paris*, **192**, 1284.
—— (1934). *C.R. Soc. Biol., Paris*, **115**, 1168.
Ferry & Levy (1934). *J. biol. Chem.* **105**, xxvii, *Proc.*
Lewis & Wells (1927). *J. infect. Dis.* **40**, 317.
Neuberger (1938). *Biochem. J.* **32**, 1435.
Sevag & Seastone (1934). *Z. ImmunForsch.* **83**, 464.

19. OBSERVATIONS ON THE STRUCTURE OF THE BARIUM SALT OF HEPARIN

By A. F. CHARLES and A. R. TODD

From the Chemistry Department, University of Manchester, and the Connaught Laboratories, University of Toronto

(*Received 1 December 1939*)

The question of the nature of heparin, an inhibitor of blood coagulation in mammals, has engaged the attention of various workers since its discovery in the liver of dogs by Howell & Holt [1918]. The early preparations of heparin were very impure but Howell [1928] examined a fairly pure material and considered it to be a glycuronic acid derivative. Charles & Scott [1933] were able to confirm the carbohydrate nature of heparin although they were unable to obtain a positive test for glycuronic acid; they noted further that their most active preparations contained some 2% of nitrogen. A valuable series of investigations by Jorpes [1935] and Jorpes & Bergström [1936] clarified the position considerably as their evidence indicated that heparin might be regarded as containing polysulphuric esters of a mucoitin. Jorpes & Bergström were able to isolate glucosamine on hydrolysis of heparin, while the amount of CO_2 evolved on heating with strong HCl indicated the presence of a large proportion of uronic acid residues. It should, however, be borne in mind that although the uronic acid concerned is probably glycuronic acid its identification as such is not rigid, and actual isolation of the acid in question from heparin would be desirable. The isolation of a crystalline barium salt of heparin by Charles & Scott [1936] represented a marked advance, since a great deal of the confusion existing in the literature on heparin is doubtless due to the varying degrees of purity of the amorphous preparations used by different workers.

The work described in this paper has been undertaken with the object of confirming the homogeneity of the crystalline barium salt and of obtaining further insight into its chemical constitution.

Experimental

Barium salt of heparin

Several methods for the purification of heparin have been developed in the Connaught Laboratories, and these have been used to prepare material both from beef liver and beef lung suitable for conversion into the barium salt. The crude heparin was prepared according to the method of Charles & Scott [1934] and purified by either of the following methods.

I. Crude heparin (*ca.* 150 g.) is dissolved in alkaline water (5 l.), the acidity adjusted to *p*H 5 with acetic acid and an aqueous solution of $CdCl_2$ run in until no further precipitation occurs. The mixture is warmed to 60° and filtered. To the filtrate NaCl is added to 0·85% and the heparin precipitated by the addition of 95% alcohol (2 vol.).

II. Crude heparin (*ca.* 150 g.) dissolved as above in alkaline water is treated with a solution of $(NH_4)_2CO_3$ (1200 ml. of 20%), warmed to 60° and allowed to

stand several hours. After removal of the dark brown precipitate by centrifuging, the filtrate is made just acid to litmus and animal charcoal (60 g.) added. The mixture is warmed to 60°, stirred 1 hr. and allowed to stand 4–5 hr. before filtering. The filtrate, to which 1 % of acetic acid is added, is again treated with charcoal, and the process repeated until the filtrate is practically colourless. To the final solution NaCl is added to 0·85 % and the heparin precipitated as above with alcohol.

In preparing the barium salt it has been found most satisfactory first to prepare the ammonium salt, which can readily be obtained with an ash content $<1\,\%$. Its preparation may be carried out in two ways.

A. Heparin (10 g.) purified by either of the above methods is dissolved in ammoniacal water (125 ml.) and $(NH_4)_2CO_3$ solution (15 ml. of 20 %) is added. After warming to 60° the mixture is allowed to cool and filtered. From the clear filtrate the heparin salt is precipitated by the addition of acetic acid (9 vol.), collected, washed with alcohol and ether and dried.

B. Benzidine method. This has already been described [Charles & Scott, 1936].

Conversion of the ammonium salt into the crystalline barium salt is carried out as already described by Charles & Scott [1936]. In the present work the barium salt was prepared from heparin isolated from lung and liver by the above methods and was analysed for potency, ash content and nitrogen. The results are shown in Table 1, the potency being determined by the method of Charles & Scott [1936].

Table 1

Starting material	Methods	% ash	Potency u./mg.	% N
Beef lung	$CdCl_2$-$(NH_4)_2CO_3$	33·1	98	2·13
,,	$CdCl_2$-benzidine	33·8	98	2·04
,,	Charcoal-benzidine	33·7	104	1·89
,,	Charcoal-$(NH_4)_2CO_3$	33·1	97	1·90
Beef liver	$CdCl_2$-$(NH_4)_2CO_3$	33·8	100	2·20
,,	$CdCl_2$-benzidine	33·2	98	2·01
,,	Charcoal-$(NH_4)_2CO_3$	33·3	98	1·90

Mr L. B. Jaques of the Connaught Laboratories has prepared in similar fashion from beef intestine a crystalline barium salt apparently identical with the products from lung and liver; it had an ash content of 33·4 %, contained 1·89 % N and had a potency of 100 u./mg.

These findings indicate that the barium salts prepared by various routes from different beef tissues are identical and support our view that the salt is a chemical individual.

From the barium salt, by treatment with $(NH_4)_2CO_3$ followed by filtration from $BaCO_3$ and precipitation with acetic acid, the amorphous ammonium salt may be prepared; this material contains 0·5 % or less of ash and biological tests indicate a 90 % recovery of activity; the balance is possibly adsorbed on the $BaCO_3$. The results of a series of analyses on these salts indicate that heparin contains 5 S atoms for every 2 N atoms, a result in agreement with earlier observations [Charles & Scott, 1936] and with the findings of Reinert & Winterstein [1939]; the analytical values are discussed later in this paper.

The ammonium salt of heparin was reconverted into the barium salt in the usual way. After collecting the first crop of crystals a second fraction was obtained by cooling in the ice-chest; the residual material in the mother liquor was isolated by evaporation *in vacuo*, and washing free of barium acetate with 80 % acetic acid. The sulphur content of the various products was determined.

The results collected in Table 2 show that the ammonium salt can be converted into a barium salt of uniform sulphur content, differing only in physical appearance due to mode of separation.

Table 2

Substance	Weight (g.)	S %	S content (mg.)
Ammonium salt*	2·00	12·2	244
Ba salt, 1st crop	1·77	9·6	170
Ba salt, 2nd crop	0·17	9·8	17
Ba salt from mother liquor	0·22	9·6	21

* The ammonium salt used was dried at room temperature.

The claim of Jorpes & Bergström [1939] that heparin is a mixture of sulphuric esters which can be separated by fractionation with brucine into several fractions with varying sulphur content prompted us to apply the process to the crystalline barium salt. Using either brucine or brucine sulphate the results were negative; both brucine precipitates and supernatants on working up gave material of the same sulphur content and all could be reconverted into a barium salt apparently identical with the starting material.

Condition of nitrogen in heparin. Heparin yields glucosamine on hydrolysis and it is virtually certain that all the nitrogen in the molecule is accounted for by amino-sugar residues. While it would be expected that the amino-groups would be acetylated as in, for example, chondroitin sulphuric acid, small amounts (up to *ca.* 0·5 %) of nitrogen are liberated from heparin in normal Van Slyke estimations. In view of the observation of Hynd & Macfarlane [1926], who found that quantitative evolution of nitrogen from glucosamine under the usual Van Slyke conditions was only attained after some 16 hr. shaking, we felt it was desirable to apply the same technique to heparin. Dr C. P. Stewart of Edinburgh University, to whom we are deeply indebted, carried out for us parallel determinations on *N*-acetylglucosamine (kindly supplied by Dr W. T. J. Morgan) and on the barium salt of heparin. The results showed that the amino-N value for heparin increased gradually with the time of reaction until after 16 hr. the value reached (1·84 %) represented virtually all the N present. Under the same conditions *N*-acetylglucosamine gave no measurable amount of nitrogen even in 16 hr.

A series of *N*-acetyl determinations were made by three different analysts but the results obtained even with the same sample were not concordant (Found: *N*-acetyl 1·26, 1·56; 1·76, 2·9; 2·2). It is difficult to lay much stress on these values although they indicate that part at least of the N in heparin is acetylated. The curious result of the Van Slyke determinations is doubtless due to slow hydrolysis of the *N*-acetyl grouping.

Inactivation of heparin with acids

That heparin loses its potency when treated with alcohol containing small amounts of HCl has already been reported [Charles & Scott, 1936]. This inactivation is attended by loss of sulphur-containing groups, and by varying the acid and alcohol concentration and the temperature and duration of reaction a correlation between sulphur content and activity has been obtained. The results are collected in Table 3. In each case the products were precipitated as amorphous barium salts and the ash content taken as a measure of sulphur content. Potency is expressed relative to the barium salt of heparin.

Table 3

Product	*N* HCl conc.	% MeOH	Temp.	Time	% ash	Potency
Heparin Ba Salt	—	—	—	—	33·5	100
1	0·01	25	5°	1 hr.	31·2	72
2	0·01	50	5°	1 hr.	30·5	57
3	0·025	50	5°	1 hr.	28·6	43
4	0·05	50	5°	1 hr.	27·9	40
5	0·1	99	5°	10 min.	26·0	22
6	0·1	99	5°	20 min.	24·6	20
7	0·028	99	5°	17 hr.	24·5	20
8	0·1	99	5°	1 hr.	22·4	13
9	0·05	99	5°	17 hr.	21·0	9
10	0·1	99	5°	2 hr.	20·1	8
11	0·1	99	5°	17 hr.	18·0	4
12	0·1	99	20°	1 hr.	15·2	2·7
13	0·1	99	20°	2 hr.	12·7	1·5
14	0·1	99	20°	7 hr.	8·5	1·0
15	0·1	99	20°	17 hr.	7·4	0·6

These results indicate that the potency varies with the sulphur content of the products.

With a view to obtaining an insight into the nature of the above inactivation the ammonium salt of heparin (1 g.) was treated with 0·1 *N* HCl in 99 % MeOH at 20° during 24 hr. The reaction products were then precipitated by addition of acetic acid (9 vol.), collected, washed with ether and dried. Analysis of the material obtained showed that approximately 80 % of the S had been removed, although the remainder appeared still to be present in an ammonium salt the NH_4-N/S ratio being 1/1. The methoxyl content was 7 % which is probably significant since heparin dissolved in MeOH and reprecipitated showed no methoxyl content. The inactivated product was not obtained in crystalline form and is certainly not a single substance; unlike heparin, it had definite though weak reducing properties, and was at least partially dialysable. Further work is in progress on the nature of this material but its properties indicate that some breakdown of the carbohydrate molecule takes place in the treatment with acidic alcohol. This may explain why attempts to re-introduce sulphate groups into the inactivated material failed to yield any product with significant physiological action even although materials containing as much as 8 % S were obtained.

Experiments were also carried out in which heparin was hydrolysed by heating with *N* H_2SO_4, hydrolysis being continued until the solutions had a reducing value corresponding to half that expected for complete hydrolysis to monosaccharide derivatives. So far it has not been possible to obtain any crystalline identifiable product from these experiments, possibly because of the small amounts of material employed.

Oxidation experiments

The use of periodic acid as a specific oxidizing agent for α-glycols introduced by Malaprade [1928] has recently been applied by Hudson and his collaborators [cf. Jackson & Hudson, 1937] in an elegant series of investigations on sugar derivatives. Application of this reagent offers the possibility of learning something of the distribution of sulphuric acid groupings in heparin and we have begun a series of investigations to this end. Preliminary experiments show that heparin itself is stable to periodic acid while heparin inactivated by various processes shows varying degrees of reducing power towards this reagent. The results of the investigations will be reported later.

Discussion

It has been shown that the crystalline barium salt of heparin prepared by various methods from different beef tissues has uniform composition and physiological activity. It can be converted into an ammonium salt and the latter reconverted almost quantitatively into a barium salt with the same composition as before. Moreover, the heparin from the barium salt showed no evidence of separating into distinct fractions with brucine. The ratio S/N in heparin appears to be 5/2 on the basis of analyses of the barium and ammonium salts. The same ratio has recently been found by Reinert & Winterstein [1939] for the sodium salt.

In a recent publication Jorpes & Bergström [1939] cast doubts both on the crystalline nature and on the homogeneity of the barium salt without apparently having themselves examined it. Of its crystalline nature there can be no doubt; we had hoped to obtain some insight into the detailed structure of the salt by X-ray examination but Dr W. H. Taylor of the Manchester College of Technology found that the crystals were too small and heavily twinned for the X-ray diagram to be of much assistance; we are indebted to Dr Taylor for examining the material. As regards homogeneity we consider that all the available evidence suggests that the barium salt is a chemical individual; the possibility that we are dealing with mixed crystals seems unlikely in view of the various interconversions carried out without change in composition. In the communication of Jorpes & Bergström [1939] there appears the remarkable statement that the ratio S/N = 2·5/1 in the formula suggested by Charles & Scott [1936] for the barium salt "immediately disproves its correctness". Since the ratio might equally well be written 5/2 and there is no evidence to show that heparin is built of a recurring fundamental unit of 1 mol. glucosamine and 1 mol. uronic acid, each unit containing the same number of sulphate residues, we are unable to see the force of the argument presented. As regards sulphur content it should be noted that heparin preparations contain varying amounts of water according to the drying procedure adopted; this may explain the varying sulphur content quoted by different workers although it should not affect the more significant S/N ratio.

The brucine fractionation experiments of Jorpes & Bergström [1939] carried out on impure amorphous heparin preparations are interesting, although some separation was to be expected. One striking feature emerges on analysis of the figures published by these authors; the recovered material accounts for 70 % of the weight and 70 % of the S of the heparin used, while the unrecovered material accounts for some 30 % of the weight and about 50 % of the original activity. Thus, if no degradation of the active heparin has occurred in the experiment, the material lost in the brucine fractionation has an activity per unit weight equal to that of the most active material isolated. Equally striking is the low sulphur content of the material which has apparently been lost. It is doubtless true that crude heparin may consist of a mixture of compounds of varying sulphur content, but this does not in any way reflect on the homogeneity of the barium salt isolated from it.

As a result of the investigations so far carried out on heparin one may consider it as a mucoitin polysulphuric acid. Accepting as a basis the mucoitin formula of Levene [1925] and regarding as a fundamental unit a tetrasaccharide structure made up of 2 mol. *N*-acetylglucosamine and 2 mol. glycuronic acid it is clear that each unit must contain 5 sulphuric acid residues, i.e. heparin should have the formula

$$C_{22}H_{29}O_{12}\begin{cases}\text{-}(OSO_3H)_5\\ \text{-}(COOH)_2\\ \text{-}(NHCOCH_3)_2\end{cases}$$

This formula has also been proposed by Reinert & Winterstein [1939] who have shown that the analytical values of the sodium salt are in accordance with it. The crystalline barium salt has been analysed on numerous occasions, and contains 5 Ba atoms to every 10 S atoms; since the sodium salt of Reinert & Winterstein [1939] contains 7 Na atoms to every 5 S atoms one must conclude that the carboxyl groups in the barium salt are free or that they are lactonized, i.e. that the glycuronic residues are in the form of glycuronolactone. We have been unable to demonstrate the presence of lactone groupings although the mode of preparation of the barium salt would not conflict with their presence; the presence of free carboxyls is supported by the fact that a 1% aqueous solution of the salt has *p*H *ca.* 3·3 (calomel electrode) and that it readily takes up approximately 2 equivalents of NaOH in the cold. On this view the formula of the barium salt should be $(C_{28}H_{39}O_{38}N_2S_5)_2Ba_5$. Allowing for $24H_2O$ in the hydrated crystals the analytical values are in fairly good agreement with the calculated values. (Found: Ba, 19·7; N, 1·9; S, 9·6; H_2O (loss at 60° in high vac.), 12·1 $(C_{28}H_{39}O_{38}N_2S_5)_2Ba_5$ $24H_2O$ requires Ba, 19·8; N, 1·6; S, 9·3; H_2O, 12·5. Found in anhydrous salt C, 20·2; H, 3·5. Required C, 22·5; H, 2·6. Charles & Scott [1936] found in hydrated salt C, 18·0; H, 3·9. Required C, 19·3; H, 3·6.

Treatment of the barium salt with $(NH_4)_2CO_3$ gives an amorphous ammonium salt whose analysis is also in agreement with the heparin formula under discussion. It would appear to contain either 5 or 6 (NH_4) radicles to every 5 S atoms. (Found (anhydrous): NH_4-N, 6·1; bound N, 2·3; S, 12·0, $C_{28}H_{39}O_{38}N_2S_5(NH_4)_5$ requires NH_4-N, 5·6; bound N, 2·2; S, 12·7. $C_{28}H_{38}O_{38}N_2S_5(NH_4)_2$ requires NH_4-N, 6·6; bound-N, 2·2; S, 12·5.)

Assuming that there are two free carboxyl groups in the barium salt the formula containing 6 (NH_4) radicles could be understood on the grounds that the second carboxyl might be too feeble to form an (NH_4) salt under the conditions employed. It can, of course, be readily understood that the sodium salt of heparin formed by means of NaOH will contain 7 Na atoms to every 5 S atoms.

It should be realized that although the analytical values obtained are in agreement with the formula proposed, they do not constitute proof of its correctness. It is wellnigh impossible to deduce an accurate empirical formula of this magnitude on the basis of analysis of salts containing some 33% of ash. The formula is however in accordance with the known facts about heparin and the analytical values do not conflict with it. Whether or not the molecular formula of heparin corresponds to the simple tetrasaccharide structure, or whether it is a complex formed by fusion of a number of such units cannot be stated definitely at present.

The question of a standard heparin preparation is discussed by Jorpes & Bergström [1939]. It seems reasonable to suppose that the most satisfactory standard would be the stable crystalline Ba salt rather than amorphous preparations from liver whose activity admittedly varies.

Summary

1. Various methods are described for the preparation of a crystalline barium salt of heparin.

2. Evidence is presented for the view that the barium salt, isolated from various tissues, is identical in properties and is a chemical individual.

3. By treatment with acidified methyl alcohol under varying conditions heparin yields a series of products of lower physiological activity, decrease in activity being accompanied by a decrease in sulphur content. Material completely

inactivated in this way appears to have undergone structural alteration in addition to removal of sulphate groupings.

4. The structure of heparin is discussed. The results of investigations to date are best explained on the basis that it is a mucoitin sulphuric acid in which the basic tetrasaccharide unit contains 5 sulphuric ester groupings. Analyses of the barium salt and of the ammonium salt prepared from it are in agreement with this view. In the barium salt the carboxyl groups appear to be unsubstituted.

REFERENCES

Charles & Scott (1933). *J. biol. Chem.* **102**, 425.
—— —— (1934). *Trans. roy. Soc. Can.* **28**, 49.
—— —— (1936). *Biochem. J.* **30**, 1927.
Howell (1928). *Johns Hopk. Hosp. Bull.* **42**, 199.
—— & Holt (1918). *Amer. J. Physiol.* **47**, 328.
Hynd & Macfarlane (1926). *Biochem. J.* **20**, 1264.
Jackson & Hudson (1937). *J. Amer. chem. Soc.* **59**, 994.
Jorpes (1935). *Biochem. J.* **29**, 1817.
—— & Bergström (1936). *Hoppe-Seyl. Z.* **244**, 253.
—— —— (1939). *Biochem. J.* **33**, 47.
Levene (1925). Hexosamines and Mucoproteins, p. 84.
Malaprade (1928). *C.R. Acad. Sci. Paris*, **186**, 382.
Reinert & Winterstein (1939). *Arch. int. Pharmacodyn.* **62**, 47.

20. THE SPECIFICITY OF DIAPHORASE (COENZYME FACTOR)

By EDWARD PENLEY ABRAHAM and ERICH ADLER

From the Biochemical Institute, University of Stockholm

(*Received 16 October 1939*)

The independent discovery of diaphorase by Adler *et al.* [1937, 1] and by Dewan & Green [1937], who used the name coenzyme factor, was accompanied by disagreement with regard to the specificity of this enzyme. Dewan & Green [1938], on the basis of Thunberg experiments with the Robison ester system, using skeletal muscle as a source of the coenzyme factor, maintained that it reacted with the reduced form of codehydrogenase II (Co II) as well as that of codehydrogenase I (Co I, cozymase). Euler & co-workers [1938] and Adler *et al.* [1939, 3], employing the spectrophotometric technique and using heart muscle and yeast as starting materials, stated that partially purified preparations of diaphorase reacted only with reduced Co I. Later experiments by Adler *et al.*, however [1939, 2], indicated that the diaphorase action of crude aqueous extracts from acetone-dried tissues depended on the organ used as starting material. While, in the case of heart, the reaction with Co II was very much slower than that with Co I, in the case of adrenal gland the two reactions were of comparable velocity. Liver occupied an intermediate position. These results were explained most simply by assuming the existence of two diaphorases, the first of which acted specifically with Co I while the second was either specific for Co II or else acted with both coenzymes. These were called diaphorase I and diaphorase II respectively.

Recently Straub [1939] has obtained from heart muscle a pure flavoprotein, which Corran *et al.* [1939] showed was almost certainly identical with the heart coenzyme factor. The pure preparation was stated to be active with both coenzymes, but the reaction with Co II was not studied in detail. The relation of the coenzyme factor to the yeast flavoprotein of Haas [1938], which was also said to react with both coenzymes, is not at present clear.

The experiments presented here, which were designed to elucidate the question of specificity, clearly support the view that there are two diaphorases. One of these, which acts only with Co I, occurs in preparations from heart muscle practically unaccompanied by the other.

Experimental

Spectrophotometric measurements. These were kindly carried out by Mr G. Günther. The rate of reaction in the system: reduced coenzyme + diaphorase + methylene blue + O_2 was measured by decrease in the absorption at 334 mμ in the usual manner. The coenzyme solution consisted of a mixture of Co I and Co II, prepared enzymically from cozymase by the method of Adler *et al.* [1939, 1] and reduced before use by hydrosulphite.

Thunberg experiments: (*a*) the diaphorase I activity of solutions was measured from the reduction time of methylene blue in the system: CoH_2 I + diaphorase + MB. In each tube were used: 0·2 ml. CoH_2 I (100 γ), 0·25 ml. $M/2$ phosphate

*p*H 7·6, 0·25 ml. MB (1 : 5000), an appropriate quantity of the solution to be tested and water to bring the total volume to 1·5 ml. It should be noted that the non-enzymic reduction of MB by CoH_2 I is very strongly catalysed by red light [cf. Adler *et al.* 1937] and that it is therefore essential for the thermostat to be shielded from bright daylight.

(*b*) An attempt was first made to measure the diaphorase II activity of solutions by the use of the Robison ester dehydrogenase system.[1] This was abandoned when it was found that some tissue extracts, especially extracts from liver, contained a strong inhibitor of the reaction. Experiments were therefore made with the *iso*citric dehydrogenase system,[2] which Adler *et al.* [1939, 4] had found to require Co II as the specific coenzyme. In each tube were used: 0·1 ml. *iso*citrate solution, 0·25 ml. *iso*citric *apo*dehydrogenase solution (18 mg./ml. of "enzyme C" from heart muscle, practically free from diaphorase [cf. Adler *et al.* 1939, 4], 0·25 ml. Co II (12·5 μg.), 0·2 ml. of a solution containing Mn^{++} ($5\times10^{-3}M$) and Mg^{++} ($2{\cdot}5\times10^{-2}$ *M*), 0·25 ml. veronal-acetate buffer (Michaelis) *p*H 7·66, 0·5 ml. MB (1 : 5000), an appropriate quantity of the solution to be tested and water to make the total volume up to 2·0 ml. The Co II was prepared enzymically from Co I [Adler *et al.* 1939, 1]. In this system the inhibition referred to above occurred in some cases when the concentration of the *iso*citrate solution used was *M*/50, but it was almost completely abolished by increasing this concentration to *M*/10.

All diaphorase activities measured by Thunberg experiments were controlled by comparison with the activity of a standard "old" flavin enzyme solution.

Preparation of extracts from acetone-dried tissues

Extracts were prepared from heart, liver and adrenal gland as follows. The fresh material was freed from fat and connective tissue, minced, and then dried by shaking twice with four times its volume of acetone and subsequent exposure to air in a thin layer. 5 g. of the dry powder were then ground with 2·5 g. sand and 25 ml. water for $\frac{1}{2}$ hr. and the resulting mixture pressed through muslin and centrifuged. The solution was dialysed for 20 hr. against running tap-water and the precipitate which formed centrifuged off. In this manner clear reddish solutions were obtained from heart and liver (12 and 17 ml. respectively). The solution from adrenal gland (12 ml.) could not be centrifuged clear.

Partial purification of heart and adrenal gland diaphorase by the method of Straub [1939]

In the case of heart this purification was carried as far as the elution from alumina Cγ. The eluate was dialysed against running tap-water for 40 hr., brought to *p*H 7·5 with *N*/10 NaOH and centrifuged. From one pig heart there were obtained 30 ml. of a clear yellowish solution.

The purification of adrenal gland diaphorase was carried out as follows: 700 g. adrenal gland were freed from fat and minced. The mince was washed 10 times with 500 ml. of water, the liquid being pressed out gently through muslin. The residue (430 g.) was ground for 1 hr. with sand and 600 ml. of *M*/50 Na_2HPO_4 (added in small portions); 200 ml. water were then added and the mixture centrifuged. To the resulting solution were added 25 ml. of *M*/1 acetate buffer *p*H 4·5. The precipitate was centrifuged off, suspended in 400 ml. water + 8 g.

[1] In this connexion it may be noted that extracts from adrenal gland contain a strong, Co II-specific, Robison ester dehydrogenase.

[2] We are grateful to Dr C. Martius, of Tübingen, for a gift of *iso*citric acid.

$(NH_4)_2SO_4$ + 12 ml. ethyl alcohol and the suspension heated 15 min. at 43°. After cooling and centrifuging the solution was dialysed for 18 hr. Adsorption was then effected by the addition of two 15 ml. portions and then one 10 ml. portion of alumina Cγ (1·7 %). The remaining solution had a weak brownish-yellow colour. Elution from the alumina was carried out with 12 ml. portions of $M/5$ Na_2HPO_4 and the eluate dialysed for 40 hr. against running tap-water. The solution was brought to *p*H 7·5 with $M/10$ NaOH and the small white precipitate centrifuged off. This procedure gave 140 ml. of a clear yellowish solution.

Results

Spectrophotometric investigations with diaphorase solutions from heart and adrenal gland

When it had been established that in Thunberg experiments certain tissue extracts inhibited the reaction designed to measure the activity of diaphorase II the possibility was considered that such an inhibition was responsible for the Co I specificity, previously reported from this Institute, of diaphorase solutions prepared from heart and yeast. Fig. 1 shows clearly that this is not the case.

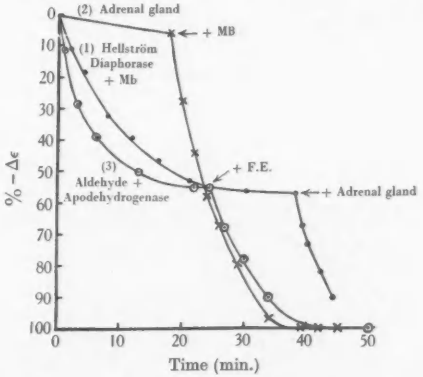


Fig. 1. Spectrophotometric experiments. Oxidation of a mixture of CoH_2I and CoH_2II. Curve (1) corrected for non-enzymic reaction between CoH_2I and Mb [cf. Adler *et. al.* 1939, 3].

Curve (3) shows the relative proportions of CoH_2 I and CoH_2 II in the coenzyme solution used, aldehyde in the presence of alcohol *apo*dehydrogenase oxidizing only the CoH_2 I and the remaining CoH_2 II being subsequently oxidized by the addition of "old" flavin enzyme (F.E.). Curve (2) shows the effect of using an extract of acetone-dried adrenal gland as a source of diaphorase. It is evident, (*a*) that there is very little aerobic reaction in the absence of MB, and (*b*) that the diaphorase transfers hydrogen from both reduced coenzymes to MB. Curve (1) shows that diaphorase prepared from heart by the method of Euler & Hellström [1938] transfers hydrogen only from reduced Co I, but that on addition of adrenal gland extract CoH_2 II also reacts. The difference in the diaphorase actions of the solutions obtained from heart and adrenal gland is therefore not due to the presence of an inhibitor in the former solution.

Comparison of the diaphorase I and diaphorase II activities of acetone-dried tissue extracts by the Thunberg technique

In all the following Figs. the diaphorase activity is expressed in the form $(I/T - I/T_0) \times 100$. T is the time, in minutes, of MB decoloration in the presence of the given diaphorase solution and T_0 is the corresponding time in the absence of diaphorase. The activity is plotted against the volume, in ml., of the solution used. In each case the "old" flavin enzyme solution used as a control is of the same concentration.

In Table 1 are given figures relating to the diaphorase I activities of extracts from liver, adrenal gland and heart. F.E. denotes the standard "old" flavin enzyme solution.

Table 1

Diaphorase I

Enzyme	T (min.)	$(I/T - I/T_0) \times 100$
0·2 ml. F.E.	2·75	34
0·1 ml. F.E.	5·0	18
0·05 ml. F.E.	9·0	9
0·025 ml. F.E.	16·0	4
0·1 ml. liver	4·0	23
0·05 ml. liver	7·75	12
0·025 ml. liver	14·0	5
0·1 ml. ad. gland	2·75	34
0·05 ml. ad. gland	5·0	18
0·025 ml. ad. gland	9·0	9
0·1 ml. heart	1·66	58
0·05 ml. heart	3·25	29
0·025 ml. heart	6·0	15
—	60 ($=T_0$)	0

Fig. 2 shows the relative diaphorase II activities of the same solutions, measured in the *iso*citric acid dehydrogenase system. In this system the decoloration time without diaphorase (T_0) was 80 min. The increase in activity on adding flavin enzyme to experiments already containing solutions from heart and adrenal gland indicates that there is no inhibition of the reaction, but in the case of liver a small inhibition appears to occur. From a consideration of Table 1 and Fig. 2 an approximate estimate can be made of the ratio, diaphorase I activity/diaphorase II activity, in the three solutions. These are as follows:

Adrenal gland	4·5 : 1
Liver	20 : 1
Heart	130 : 1

The value for liver is a maximum as a certain amount of inhibition of the *iso*citric dehydrogenase appears to occur in this case.

The values in Fig. 2 were obtained by the use of $M/10$ *iso*citrate (0·1 ml.) in the test system. Fig. 3 shows the effect of employing $M/50$ *iso*citrate. It is apparent that, while there is still no inhibition with adrenal gland, in the case of liver this has become very marked. With the solutions obtained from heart the inhibition was so strong that all values of $(I/T - I/T_0)$ were negative: for this reason they are not shown.

In the main the results described in this section confirm those previously obtained by Adler *et al.* [1939, 2], with extracts from different acetone-dried tissues, using the spectrophotometric technique.

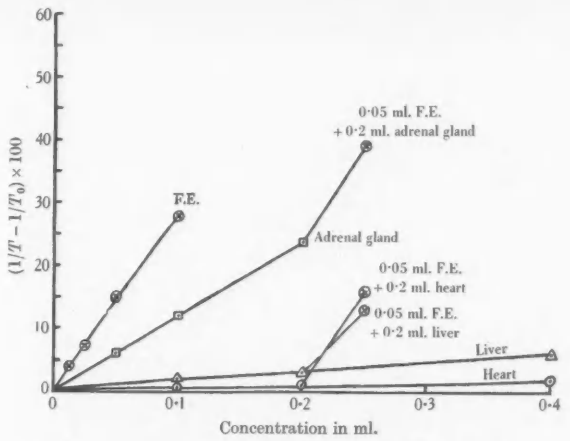


Fig. 2. Thunberg experiments. Extracts from acetone-dried organs. Diaphorase II activity (*M*/10 *iso*citrate).

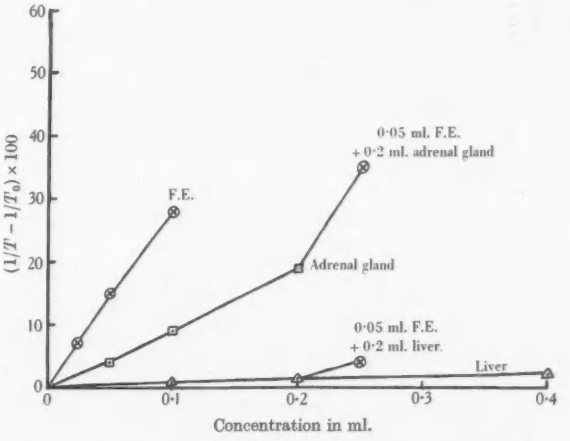


Fig. 3. Thunberg experiments. Extracts from acetone-dried organs. Diaphorase II activity (*M*/50 *iso*citrate).

Comparison of the diaphorase I and diaphorase II activities of heart and adrenal gland extracts partially purified by the method of Straub [1939]

In view of the fact that the pure flavoprotein of Straub was said to be active with both coenzymes it was considered of interest to compare the diaphorase I and diaphorase II activities of extracts from heart and adrenal gland which had been partially purified by the same procedure. The purification has already been described.

The relative diaphorase I and diaphorase II activities are shown in Figs. 4 and 5. In both cases there was no inhibition in the *iso*citric dehydrogenase system when $M/50$ *iso*citrate was used. It is evident that the diaphorase II

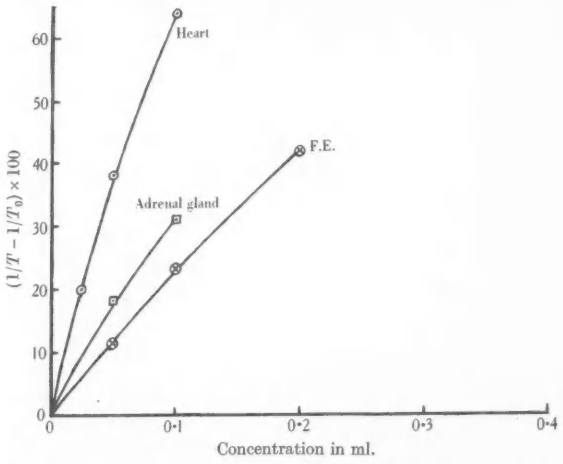


Fig. 4. Thunberg experiments. Heart and adrenal gland extracts partially purified by the method of Straub. Diaphorase I activity.

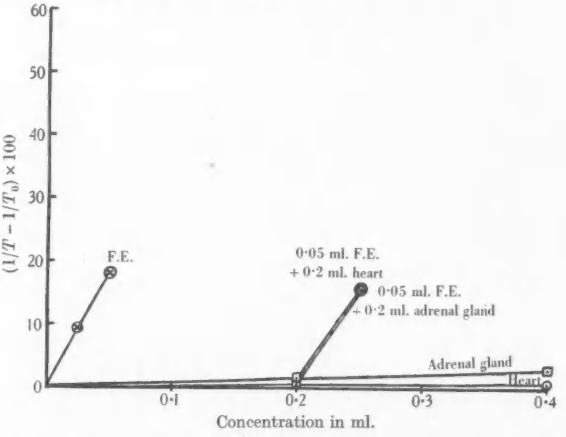


Fig. 5. Thunberg experiments. Heart and adrenal gland extracts partially purified by the method of Straub. Diaphorase II activity ($M/50$ *iso*citrate).

activity of the solution from heart is negligible, while in the case of adrenal gland it is very much smaller, relatively, than that of the extract from acetone-dried adrenal gland powder. In the present case the ratio, diaphorase I activity/diaphorase II activity, was approximately 180 : 1. It thus appeared that in

some step of the purification the diaphorase II had been almost entirely removed. This was not due to the removal of a heat-stable substance: addition of acetone-dried adrenal gland extract, which had been heated to 80° for 5 min., had no effect on the activity.

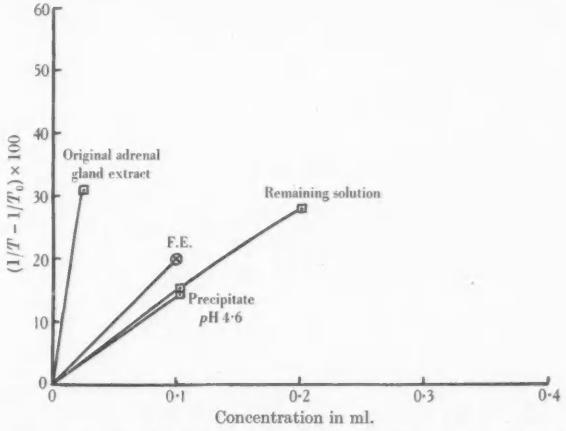


Fig. 6. Thunberg experiments. Acetone-dried adrenal gland extract; precipitate by acetate buffer *p*H 4·5, and remaining solution. Diaphorase I activity.

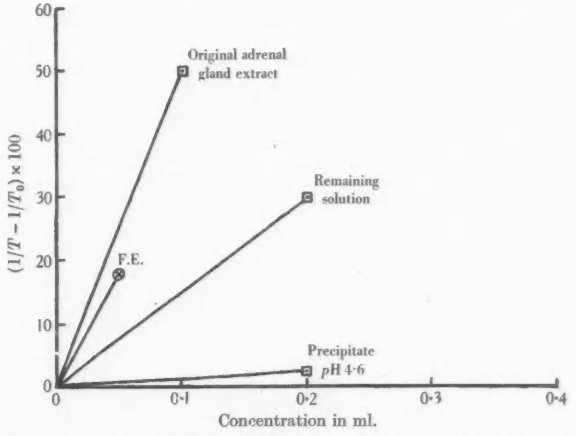


Fig. 7. Thunberg experiments. Acetone-dried adrenal gland extract; precipitate by acetate buffer *p*H 4·5, and remaining solution. Diaphorase II activity (*M*/50 *iso*citrate).

*Effect of precipitation of acetone-dried adrenal gland extract by acetate buffer p*H *4·5*

With the above results in mind it was decided to repeat the purification procedure with the extract from acetone-dried adrenal gland known to be active with respect to diaphorase II. To 8·5 ml. of such a solution was added 0·2 ml.

of $M/1$ acetate buffer pH 4·5. The precipitate was centrifuged off, suspended in water and brought to pH 7·5 by $N/10$ NaOH (vol. 10 ml.). The remaining solution was brought to pH 7·5 by $N/10$ NaOH and the volume made up to 10 ml. These two solutions were tested in the usual manner and compared with the original solution before precipitation. Figs. 6 and 7 show that, while the diaphorase I activity was approximately evenly divided between the precipitate and the remaining solution, the diaphorase II activity was associated almost entirely with the solution. This indicates the explanation of the results reported in the previous section. In the present case the ratios, diaphorase I activity/diaphorase II activity, are approximately as follows:

Original dialysed extract	4·5 : 1
Solution remaining after precipitation with acetate buffer	1·8 : 1
Precipitate resuspended	180 : 1

Although a considerable loss of both diaphorase I and diaphorase II activity occurred during the precipitation a solution was obtained by this procedure in which the ratio of the diaphorase II to diaphorase I activity was more than doubled.

On precipitating the last solution with $(NH_4)_2SO_4$ at 0·45 and 0·8 saturation (pH 6·6) most of the diaphorase I and diaphorase II appeared in the second fraction and the activity ratios were not significantly altered.

Conclusions

Both spectrophotometric and Thunberg experiments show that the ratios of diaphorase activity, measured in systems involving Co I and Co II, differ widely with solutions prepared from different organs. These differences are real and not explicable in terms of inhibition, by certain components of the solutions, of the test system using Co II. Moreover, in the case of an extract from acetone-dried adrenal gland the above ratio can be changed considerably by precipitation with acetate buffer pH 4·5.

The only simple explanation of these facts is that there are two diaphorases. The preparation of solutions which act only with Co I shows that one of these (diaphorase I) is specific for this coenzyme. As no solution has been obtained which acts only with Co II it is not decided whether the second (diaphorase II) acts specifically with Co II or with both coenzymes. For this reason the activity ratios in the Co I and Co II systems cannot be used to make a quantitative comparison of the amounts of the two diaphorases present in the various solutions: part of the diaphorase I activity may be attributable to diaphorase II.

In agreement with previous experience in this Institute with diaphorase solutions prepared from heart muscle, the heart diaphorase partially purified by the method of Straub showed practically no activity with respect to Co II. In fact the relative diaphorase II activity appeared to be even smaller, in this case, than that of crude extracts from acetone-dried heart muscle. A preparation from adrenal gland, which had been partially purified by the same method, also showed very little diaphorase II activity, in sharp contrast to the crude extract from acetone-dried material. Further experiments with the latter suggested that these results were due to the effect of precipitation with acetate buffer pH 4·5, only diaphorase I being carried down by the precipitate.

SUMMARY

1. An investigation was made of the diaphorase activities of various solutions with respect to Co I (diaphorase I activity) and to Co II (diaphorase II activity). With the Thunberg technique diaphorase II activity was measured by the use of the *iso*citric dehydrogenase system.

2. Spectrophotometric experiments confirmed the finding that diaphorase prepared from heart according to Euler & Hellström had only diaphorase I activity, but that extracts from acetone-dried adrenal gland possessed also diaphorase II activity. It was shown that negative results with respect to diaphorase II were not due to inhibition of the test system.

3. Thunberg experiments also showed that the ratio, diaphorase II activity/diaphorase I activity, varied widely with different extracts (adrenal gland > liver > heart). In the case of adrenal gland the ratio could be changed considerably by precipitation with acetate buffer *p*H 4·5.

4. Very little diaphorase II activity could be detected in preparations either from heart or adrenal gland which had been partially purified by the method of Straub.

5. These results are considered to show clearly the existence of two diaphorases.

We are grateful to Prof. H. von Euler for his interest in this work.

One of us (E. P. A.) wishes to thank the Rockefeller Foundation for a Fellowship, held at the time of these experiments and those of a previous publication (*Biochem. J.* **33**, 543, 1939), and Prof. H. von Euler for the hospitality of his Institute.

REFERENCES

Adler & Euler (1937, 2). *Sv. Vet. Akad. Ark. Kemi*, 12 B, No. 36.
—— Elliot & Elliot (1939, 1). *Enzymologia* (in the Press).
—— Euler & Günther (1939, 2). *Nature, Lond.*, **143**, 641.
—— —— —— & Plass (1939, 3). *Skand. Arch. Physiol.* **82**, 61.
—— —— —— —— (1939, 4). *Biochem. J.* **33**, 1028.
—— —— & Hellström (1937, 1). *Sv. Vet. Akad. Ark. Kemi*, **12** B, No. 38.
Corran, Green & Straub (1939). *Biochem. J.* **33**, 793.
Dewan & Green (1937). *Nature, Lond.*, **140**, 1097.
—— —— (1938). *Biochem. J.* **32**, 628.
Euler & Günther (1938). *Naturwissenschaften*, **26**, 676.
—— & Hellström (1938). *Hoppe-Seyl. Z.* **252**, 31.
Haas (1938). *Biochem. Z.* **298**, 378.
Straub (1939). *Biochem. J.* **33**, 787.

LINDA HALL LIBRARY
KANSAS CITY, MO.

21. THE RELATIONSHIP OF BACTERIAL UTILIZATION OF CO_2 TO SUCCINIC ACID FORMATION[1]

BY HARLAND GOFF WOOD AND CHESTER HAMLIN WERKMAN

From the Bacteriology Section, Iowa Agricultural Experiment Station, Ames, Iowa, U.S.A.

(*Received 7 December 1939*)

PREVIOUS investigations by Wood & Werkman [1938; 1940] have shown that CO_2 is utilized by the propionic acid bacteria in the dissimilation of a variety of substrates and that this fixation of CO_2 is inhibited by NaF. The utilization of CO_2 in the fermentation of glycerol is accompanied by an equimolar formation of succinic acid. It has therefore been proposed that in this fermentation succinic acid formation involves the combination of CO_2 and a 3-carbon compound. The discovery that NaF inhibits CO_2 utilization provided a tool for determining the validity of the proposal.

The present report considers the influence of conditions on the utilization of CO_2 and the formation of succinic acid in the dissimilation of glycerol and glucose by the propionic acid bacteria. Especially the influence of NaF is discussed. The results show that in the dissimilation of either glucose or glycerol, inhibition of CO_2 utilization causes an approximately equimolar reduction in the formation of succinic acid.

Methods

It was necessary to determine quantitatively in a rather large number of experiments the products formed by cell suspensions acting under an atmosphere of CO_2; therefore a small volume of reaction mixture was used to reduce the required amount of cell suspension. Also the determination of CO_2 in a dissimilation under an atmosphere of CO_2 requires that changes in the volume (or pressure) of CO_2 gas above the fermentation liquor must be measured. Therefore the following technique was developed for determination of the products.

The dissimilation was carried out in a 125 ml. Erlenmeyer flask with two side cups and a ground glass joint fitted to a Barcroft-Warburg manometer having a two-way stopcock (Fig. 1). The flask was immersed in a water bath at 30° and was oscillated continuously by the usual shaking device 100 times per min. through an amplitude of 5 cm. A small mercury trap and Liebig bulb containing 5 ml. of 4*N* carbonate-free NaOH was attached to the stopcock as shown. The alkali was protected by a small soda lime tube.

The experiments were set up as follows: 24 ml. of substrate, buffer etc. were pipetted into the main chamber of the reaction flask and 4 ml. of the suspension of bacteria and 2 ml. of 10*N* H_2SO_4 into the two side cups. The flask was then attached to the manometer with rubber bands and a small piece of wood was inserted in the ground glass joint so that CO_2 could pass through. A vigorous current of CO_2 saturated with water was then directed through the stopcock into the flask for 15 min. and finally, for a few minutes through the mercury trap

[1] Journal Paper No. J-703 of the Iowa Agricultural Experiment Station. Project number 572.

to remove the air from the system. It was necessary to shake the apparatus frequently during the flushing with CO_2 in order to saturate the solutions. The apparatus was next placed in the water bath. The reading of the manometer was taken after equilibrium was established, the bacteria were tipped into the main chamber, the alkali bulb and soda lime tubes were attached and the stopcock opened to the mercury trap allowing passage of CO_2 into the alkali. At the conclusion of the experiment the 2 ml. of acid in the side cup were poured into the main chamber and after equilibrium was established the manometer was read. The difference in the initial and final readings, after correction for barometric change, multiplied by the flask-constant, equals the change in volume of the CO_2. The CO_2 in the alkali was determined as follows: the alkali was washed from the Liebig bulb, acidified with H_2SO_4, and the liberated CO_2 was absorbed in a weighed potash bulb. The CO_2 determined manometrically was added to the CO_2 of the alkali. A similar determination of CO_2 was made on a mixture to which no bacteria were added. By difference the CO_2 produced or utilized during the dissimilation was calculated.

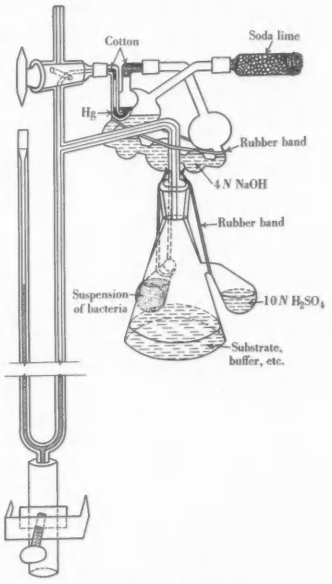


Fig. 1. Manometric apparatus.

The 28 ml. of fermented mixture plus the 2 ml. of H_2SO_4 were treated as follows.

Two ml. of the mixture were hydrolysed for 2·5 hr. in 25 ml. of boiling 3 *N* HCl and total reducing sugars were then determined according to Munson & Walker [1906] and calculated as glucose. Reducing sugars of an unhydrolysed sample were also determined. An equivalent quantity of the original cell suspension was hydrolysed for determination of its content of non-reducing sugar. The non-reducing sugar of the original cells plus that in the unhydrolysed reaction mixture was subtracted from the total sugar of the hydrolysed reaction mixture to obtain the non-reducing sugar formed by the dissimilation.

The remainder of the fermented mixture was centrifuged to remove cells. One ml. was diluted to 25 ml. and a suitable aliquot used for determination of the unfermented substrate. The glucose was determined by the method of Stiles *et al.* [1926], and glycerol as described by Wood & Werkman [1940].

Propyl alcohol is formed in the fermentation of glycerol. The alcohol was distilled from 14 ml. of the reaction mixture which was made alkaline to phenolphthalein with 10 *N* NaOH. 10 ml. of distillate were collected and oxidized with dichromate. The resulting acid was recovered by steam distillation and determined by titration (unpublished method).

The volatile acids were recovered by steam distillation from the acidified residue of the alcohol distillation in the case of glycerol fermentations. In the case of glucose fermentations 10 or 14 ml. of the centrifuged mixture were used and distilled to about 6 ml. in a micro-Kjeldahl flask. Then an additional 14 volumes (84 ml.) were collected by steam distillation. The distillate was made up to

110 ml. and the volatile acids were determined by the partition method [Osburn *et al.* 1933; 1938]. Pyruvic acid, when present, was estimated in the volatile acid distillate by the iodoform reaction [Goodwin, 1920] and a correction was made for it in the volatile acid determination. About 65 % of the pyruvic acid is distilled. HF (from NaF) also interferes; about 65 % volatilizes during steam distillation. This acid was precipitated as the Ca salt as follows. The distillate was made alkaline with $Ca(OH)_2$ and evaporated to about 2·5 ml. in a 50 ml. Erlenmeyer flask. The liquid was then filtered through a small Gooch crucible and the flask and crucible were washed with small portions of water, about 6 ml. in all. The filtrate and washings were collected in a micro-Kjeldahl flask and volatile acids were recovered from the acidified solution by steam distillation.

0·2 ml. of the reaction mixture was used for a qualitative test for pyruvic acid by the sodium nitroprusside reaction. When pyruvic acid was present 14 ml. of the reaction mixture were extracted continuously with ether for 24 hr. 10 ml. of water were added to the extract and the ether was distilled off. The solution was then made up to 50 ml. 25 ml. were used for determination of pyruvic acid and the remaining 25 ml. were treated as follows. The volatile acids were removed by steam distillation and the total non-volatile acid in the residue of distillation titrated. The succinic acid was precipitated from this solution as the Ag salt and determined by weight. Lactic acid was estimated by the method of Friedemann & Graeser [1933] on the filtrate from the succinic acid determination with the exception of those experiments in which the titration value indicated that there was no lactic acid; i.e. when the total acid was equivalent to that of the succinic and 35 % of the pyruvic and hydrofluoric acids. Pyruvic acid was never formed in the fermentation of glycerol; in this case 10 ml. of reaction mixture were extracted with ether for the succinic acid determination.

The original reaction mixture contained the following concentrations of constituents: substrate 1·60 %, $NaHCO_3$ 1·4 %, cell suspension 0·05 g. wet bacteria per ml. of reaction mixture and NaF as indicated. When phosphate buffer (0·1 *M*) was used, it was in addition to the $NaHCO_3$ and was in a molar ratio of 79 of KH_2PO_4 to 21 of K_2HPO_4. The original concentration of substrate was made up accurately by weight. The time of incubation was usually 30 hr. for glucose and 50 hr. for glycerol. No correction was made for endogenous dissimilation other than for the non-reducing sugar in the cells. On the basis of glucose this value ranged from 0·16 mg. to 1·80 mg. per ml. of reaction mixture for the different cells used and was usually about 0·30 mg.

The cells used in the reaction mixture, *Propionibacterium pentosaceum* 49W, were harvested after 5 days' growth at 30° in medium containing glucose or glycerol 0·5 %, $NaHCO_3$ 1·0 % or phosphate buffer (0·1 *M*, *p*H 6·9) and Difco yeast extract 0·4 %. The cells were washed twice with water before use.

Results shown in Table 4 were obtained with proliferating cells. The medium contained glycerol or glucose 2·0 %, $NaHCO_3$ 1·75 %, Difco yeast extract 0·4 % and NaF when indicated 0·02 *M*. In the glucose experiments (no. II in the Tables) 0·1 *M* phosphate buffer *p*H 6·15 was used in addition to $NaHCO_3$. Incubation was at 30° for 20 days in the case of glucose and 30 days in that of glycerol. The culture was 49W (*P. pentosaceum*). Procedures and methods of analysis were those described by Wood & Werkman [1938].

Experimental results

The experimental results are presented in m*M* per 100 m*M* of substrate fermented. The carbon balance and redox index (perfect index = 1·00) are for the most part satisfactory, except in those experiments where there was apparently

Table 1. *Influence of NaF on formation of succinic acid and utilization of CO_2 by cell suspension of 49 W*

Cells were grown in glycerol, yeast extract, phosphate medium

Exp. no.	Conc. of NaF in reaction mixture *M*	Phosphate in reaction mixture	Substrate	Substrate fermented per l. m*M*	Products per 100 m*M* substrate: Propionic acid m*M*	Acetic acid m*M*	CO_2 m*M*	Succinic acid m*M*	Propyl alcohol m*M*	Pyruvic acid m*M*	Non-reducing material m*M*	Carbon recovery %	O/R
II	0·000	Yes	Glycerol	161·3	46·8	3·80	−38·7	39·1	4·42	0·0	2·55	98·4	1·01
	0·0125	Yes	,,	23·8	98·6	5·52	−3·48	3·90	3·14	0·0	0·00	109·3	0·91
III	0·000	Yes	,,	158·4	60·4	4·68	−28·5	27·5	3·97	0·0	3·05	100·6	0·99
	0·015	Yes	,,	46·9	86·3	9·59	−6·67	4·25	2·75	0·0	4·60	107·6	0·97
	0·000	—	,,	159·4	65·5	4·48	−25·5	23·2	3·15	0·0	1·15	96·7	0·98
IV	0·000	—	,,	172·2	66·3	5·14	−39·0	36·7	0·00	0·0	0·0	104·7	0·95
	0·015	—	,,	108·0	97·1	4·05	−11·8	6·21	0·00	0·0	0·0	103·9	0·88
I	0·000	—	Glucose	82·6	78·7	26·6	22·0	32·3	—	—	17·9	91·3	0·97
	0·015	—	,,	64·5	130·3	13·4	54·4	14·0	—	—	5·21	93·2	0·94
II	0·000	Yes	,,	83·0	80·9	14·8	15·6	42·9	—	—	20·8	97·3	0·92
	0·015	Yes	,,	26·5	—	—	—	0·23	—	—	—	—	—
III	0·000	Yes	,,	83·0	93·0	17·1	26·1	28·7	—	4·95	17·4	95·6	0·92
	0·010	Yes	,,	69·3	120·6	17·5	51·1	13·2	—	4·73	10·7	96·6	1·00

Table 2. *Formation of succinic acid by bacteria grown in different types of media and the effect of NaF on the fermentation*

Exp. no.	Medium used for growth of cells	Conc. of NaF in reaction mixture *M*	Phosphate in reaction mixture	Substrate	Substrate fermented per l. m*M*	Products per 100 m*M* of substrate: Propionic acid m*M*	Acetic acid m*M*	CO_2 m*M*	Succinic acid m*M*	Propyl alcohol m*M*	Non-reducing material m*M*	Carbon recovery %	O/R
I	Glycerol, $NaHCO_3$, yeast extract	0·000	—	Glucose	83·0	104·8	26·7	55·6	6·87	—	10·8	85·4	1·12
		0·015	—	,,	69·8	126·6	28·6	61·8	5·85	—	6·22	93·2	1·02
II	Glucose, $NaHCO_3$, yeast extract	0·000	—	,,	83·0	101·8	27·9	49·3	7·09	—	31·2	104·3	1·04
		0·015	—	,,	83·0	118·6	27·2	61·3	7·18	—	2·68	88·2	1·09
		0·000	—	,,	109·3	74·9	5·38	−0·90	8·39	—	—	—	—
III	Glucose, phosphate, yeast extract	0·000	Yes	,,	48·0	—	—	44·7	0·0	—	17·9	—	—
		0·000	Yes	Glycerol	37·5	—	—	−6·24	4·83	—	0·0	—	—
IV	Glucose, $NaHCO_3$, yeast extract, NaF 0·02 *M*	0·000	Yes	Glucose	83·0	105·9	11·8	44·9	14·5	—	22·0	93·1	0·99
		0·020	Yes	,,	83·0	116·4	12·9	51·3	14·3	—	13·9	94·5	1·00
		0·000	Yes	Glycerol	157·5	73·9	6·25	−6·46	7·67	7·15	0·77	95·1	0·99
		0·020	Yes	,,	41·8	91·3	5·11	−3·28	4·47	4·14	0·0	103·7	0·95
		0·000	—	,,	157·4	73·4	4·84	−9·27	5·44	6·75	3·32	94·2	0·94
V	Glycerol, phosphate, yeast extract, NaF 0·02 *M*	0·000	Yes	Glucose	28·4	—	—	—	4·59	—	—	—	—
		0·020	Yes	Glucose	23·9	—	—	—	3·77	—	—	—	—

Table 3. *Influence of phosphate on formation of succinic acid by cell suspensions*

Exp. no.	PO_4 in reaction mixture	Substrate	Substrate fermented per l. m*M*	Products per 100 m*M* substrate: Propionic acid m*M*	Acetic acid m*M*	CO_2 m*M*	Succinic acid m*M*	Propyl alcohol m*M*	Pyruvic acid m*M*	Lactic acid m*M*	Non-reducing material m*M*	Carbon recovery %	O/R
I	No	Glucose	83·0	100·9	33·3	51·5	4·80	—	—	—	30·5	104·2	1·07
	Yes	,,	83·0	92·8	29·6	44·2	14·2	—	—	—	23·5	96·6	1·10
II	No	,,	83·0	102·0	18·3	43·3	1·19	—	10·5	26·4	9·72	93·2	0·96
	K_2HPO_4	,,	83·0	89·2	9·37	34·3*	6·90	—	13·7	16·2	20·5	93·6*	—
	Yes	,,	83·0	90·3	11·21	38·4	9·75	—	13·5	14·3	19·8	95·4	1·10
	No	Glycerol	158·6	82·0	5·51	−6·25	6·25	4·40	0·0	0·0	0·0	96·2	0·98
	Yes	,,	156·5	76·0	4·68	−9·61	13·0	5·82	0·0	0·0	1·28	101·6	1·00

* CO_2 not determined; value given was calculated on basis of O/R balance. Carbon recovery was calculated using the estimated CO_2 value.
I. Cells were grown in glycerol, yeast extract, $NaHCO_3$.
II. Cells were grown in glucose, yeast extract, $NaHCO_3$.

Table 4. *Influence of NaF on formation of succinic acid and utilization of CO_2 in the dissimilation of glucose*

Exp. no.	Conc. of NaF in reaction mixture *M*	Propionic acid m*M*	Difference in propionic acid m*M*	Acetic acid m*M*	CO_2 m*M*	Difference in CO_2 m*M*	Succinic acid m*M*	Difference in succinic acid m*M*	Pyruvic acid m*M*
I	0·000	94·8	41·6	32·4	26·8	30·6	39·3	−24·6	
	0·015	137·4		14·1	57·4		14·7		
II	0·000	112·6	22·5	20·7	31·6	25·6	34·8	−20·0	6·0
	0·010	135·1		19·7	57·2		14·8		5·3

Table 5. *Influence of NaF on formation of succinic acid and utilization of CO_2 by proliferating cells (49 W)*

Exp. no.	Conc. of NaF in medium *M*	Substrate	Substrate fermented per l. m*M*	Products per 100 m*M* substrate: Propionic acid m*M*	Acetic acid m*M*	CO_2 m*M*	Succinic acid m*M*	Propyl alcohol m*M*	Pyruvic acid m*M*	Lactic acid m*M*	Non-reducing material m*M*	Carbon recovery %	O/R
I	0·020	Glycerol	140·2	83·2	4·19	−5·88	5·26	1·29	0·53	0·0	0·21	93·4	1·07
	0·000	,,	212·2	67·9	2·24	−23·3	23·5	1·95	0·0	0·0	1·77	98·5	1·03
I	0·020	Glucose	103·3	116·1	13·6	51·9	7·26	—	3·15	16·1	4·95	90·7	0·98
	0·000	,,	106·3	113·9	28·0	52·1	11·3	—	1·06	3·01	6·90	91·4	1·02
II	0·020	,,	94·9	105·0	17·4	52·7	10·7	—	0·0	38·8	1·17	94·8	1·11
	0·000	,,	108·3	124·5	21·1	57·7	16·0	—	0·0	1·99	4·40	95·0	1·05

an error or all the products were not determined. Lactic and pyruvic acids were not determined in some of the earlier experiments. These products are sometimes formed, particularly from glucose; a more accurate balance might have been obtained had they been estimated.

Discussion

It is evident from a consideration of Tables 1 and 2 that the manner in which cells are grown influences their ability, as a cell suspension, to form succinic acid from glycerol or glucose. Higher yields of succinic acid were consistently obtained from cells which had been grown on the glycerol-phosphate-yeast extract medium (Table 1) than from cells grown on other types of media (cf. column 2, Table 2). The largest yield in Table 2 was 14·5 mM of succinic acid as compared with values ranging from 27·5 mM to 42·9 mM in Table 1; this, in spite of the fact that while growing on a glycerol-phosphate-yeast extract medium, the organisms produce less succinic acid than when growing on certain of the other media (e.g. glycerol-$NaHCO_3$-yeast extract). Thus the results can hardly be attributed to adaptive enzymes. It would seem that the ability of cell suspensions to form succinic acid depends upon other factors.

It appeared possible that lack of phosphate might be a limiting factor when cells were used that had been grown in a medium with no added phosphate. The results (Table 3) indicate that this explanation may in part be valid. Cells were grown in a medium to which no inorganic phosphate was added and were tested with and without the addition of phosphate. Both with glucose and glycerol the addition of phosphate increased the yield of succinic acid. In exp. I of Table 3, 4·8 mM of succinic acid were formed when phosphate was not added, and 14·2 mM when it was added. The other experiments show the same trend though the variation is not as large. The highest yield of succinic acid in these experiments, however, was by no means equivalent to that obtained with cells grown on glycerol-phosphate-yeast extract medium (Table 1). Apparently factors in addition to phosphates are involved which are inherent in the cells and determined during growth. Growth in a medium containing phosphate is not sufficient in itself to cause development of active cells since those grown in glucose-phosphate-yeast extract medium did not form much succinic acid (Table 2, exp. III). Phosphate need not be added to obtain a substantial yield of succinic acid when the cells used are from glycerol-phosphate-yeast extract medium (Table 1, exps. I and IV). In all probability sufficient phosphate is introduced with the cells to supply their requirements. The results in Table 3 show clearly that phosphate stimulates the formation of succinic acid even though large yields are not obtained. The influence of phosphate is not due to change in pH since the dibasic salt had the same effect as the more acid mixture.

The effect of cells grown in a medium containing 0·02 M NaF was determined inasmuch as Wiggert & Werkman [1939] have shown that CO_2 production from glucose by suspensions of such cells is increased in the presence of NaF. It seemed probable that the increase was caused by inhibition of the utilization of CO_2. The possibility of using cells insensitive to NaF [Wiggert & Werkman, 1939] for study of CO_2 utilization was attractive, for it is difficult when using fluoride-sensitive cells to select a concentration of fluoride such that the utilization of CO_2 is inhibited and dissimilation is not. The results obtained with cells grown in NaF (Table 2, exps. IV and V) indicate that they neither utilize much CO_2 nor produce large yields of succinic acid. The increased production of CO_2 from glucose in the presence of NaF is probably caused by suppression of the formation of non-reducing sugar. Thus more of the carbon of glucose is diverted to a gas-forming

mechanism. In all experiments of Tables 1 and 2 less non-reducing sugar was formed from glucose in the presence of NaF. There are, then, at least two ways by which NaF increases formation of CO_2; one, by inhibiting the formation of non-reducing sugars, and another, by inhibiting utilization of CO_2.

The influence of NaF on the dissimilation of glucose and glycerol, in which there was substantial formation of succinic acid and utilization of CO_2, is shown in Table 1. NaF inhibits the formation of succinic acid from both glucose and glycerol and the fixation of CO_2 in the dissimilation of glycerol. The decrease in fixation of CO_2 is accompanied by an almost identical decrease in succinic acid formation. For example, in exp. II, 38·7 mM of CO_2 were fixed and 39·1 mM of succinic acid formed in the absence of fluoride, whereas in the presence of fluoride the quantities were 3·48 and 3·90. This relationship is further evidence that the formation of succinic acid involves the utilization of CO_2. The quantitative relationships of the products formed by cell suspensions from glycerol are substantially those obtained with proliferating cells [Wood & Werkman, 1938]. Whenever the utilization of CO_2 increases, the yield of propionic acid decreases and of succinic acid increases. The amounts of acetic acid, propyl alcohol and non-reducing sugar are small and not significantly affected by the NaF or change in utilization of CO_2. Whether the decrease in propionic acid has any significance with regard to mechanism of utilization of CO_2 or is the result of diversion of the carbon to another compound cannot be answered at present. It has been established, however, that cell suspensions in the presence of propionic acid and CO_2 do not take up CO_2, nor does the addition of propionic acid to other substrates increase the utilization of CO_2.

Although the dissimilation of glucose is more difficult to explain than that of glycerol, there is no doubt that fluoride inhibits the formation of succinic acid and at the same time increases the production of CO_2. In exp. I, Table 1, fluoride addition resulted in an increased formation of CO_2 from 22·0 to 54·0 mM of CO_2 and a decrease in succinic acid from 32·3 to 14·0 mM. These changes are probably due to the inhibition of the uptake of CO_2.

It has been shown that fluoride causes an increase in the formation of CO_2 (on the basis of glucose used) by decreasing the amount of non-reducing sugar formed. Since the non-reducing sugar is assumed to be a polymer of glucose, the results can also be calculated in such a manner that the non-reducing sugar is considered as unfermented substrate. Variations due to non-reducing sugar are thus removed and the relationship of CO_2 and succinic acid is more clearly apparent (Table 4). It is evident that the main changes caused by NaF are an increase of propionic acid and CO_2 and a decrease of succinic acid. Moreover, the increased formation of succinic acid in the absence of NaF is accompanied by an almost equivalent decrease in yield of CO_2 (30·6 and −24·6 in one case and 25·6 and −20·0 in the other). This is strong evidence that CO_2 is utilized in the fermentation of glucose with formation of succinic acid as occurs in the glycerol fermentation. However, from certain experimental evidence it seems reasonable to suppose that the formation of succinic acid through utilization of CO_2 is not the only mechanism of its formation. In Table 4 if all the succinic acid had been formed by means of utilization of CO_2 there would have been produced at least 65–70 mM of CO_2 (observed CO_2 plus succinic acid), with only 14–32 mM of acetic acid. It does not seem probable that this occurred for it is likely that the 2- and 1-carbon compounds are formed initially in equimolar quantities. It is more likely that succinic acid is formed both by utilization of CO_2 and by condensation of acetic or pyruvic acid. It is noteworthy in this respect that cells obtained by growth in a NaF-glycerol-phosphate-yeast extract medium do not have a marked ability

to form succinic acid or to utilize CO_2 actively (Table 2, exp. V). Apparently the fluoride-sensitive mechanism of succinic acid formation was not developed in cells grown in the presence of NaF or in the other types of media considered in Table 2. In general the dissimilation of glucose is less sensitive to NaF than is the glycerol dissimilation and likewise the formation of succinic acid from glucose is inhibited less.

From a consideration of experimental evidence, it seems probable that formation of succinic acid by fixation of CO_2 involves the Embden-Meyerhof-Parnas mechanism of glucose or glycerol dissimilation. On the other hand, the formation of succinic acid by condensation of acetic or pyruvic acid may occur independently of the Embden-Meyerhof-Parnas reaction; i.e. by a mechanism of glucose or glycerol dissimilation which is insensitive to fluoride. Werkman *et al.* [1937] and Wiggert & Werkman [1939] have offered convincing evidence that there are at least two mechanisms of glucose dissimilation possessed by propionic acid bacteria: one sensitive to fluoride and proceeding according to the Embden-Meyerhof-Parnas scheme; the other insensitive to fluoride. It has been shown [Wood & Werkman, 1940] that phosphoglyceric acid is not dissimilated in a concentration of fluoride which inhibits utilization of CO_2. Therefore any succinic acid arising in the presence of fluoride cannot have been formed through the Embden-Meyerhof-Parnas reactions or by fixation of CO_2. Moreover, cells not possessing the mechanism for breakdown of phosphoglyceric acid but possessing the fluoride-insensitive mechanism of dissimilation (cells grown in medium containing NaF) do not fix a significant quantity of CO_2 even in the absence of NaF (Table 2, exps. IV and V). The implication is that the absence of the Embden-Meyerhof-Parnas mechanism precludes utilization of CO_2. Furthermore, phosphate slightly stimulates utilization of CO_2 (Table 3). All of these facts indicate that fixation of CO_2 involves a phosphorylating mechanism.

A few experiments were conducted to determine the effect of fluoride on dissimilations by proliferating cells. In the case of the fermentation of glycerol (Table 5, exp. I), fluoride inhibited fixation of CO_2 and formation of succinic acid as with the cell suspension. The dissimilation of glucose does not show such definite effects, possibly because of the small amount of CO_2 fixed by the proliferating cells. The formation of succinic acid was also largely uninhibited. Apparently under the conditions of these experiments glucose was fermented with only a small fixation of CO_2 and the greater part of the succinic acid was formed by acetic or pyruvic acid condensation. In the presence of fluoride considerable lactic acid was formed from glucose. This formation of lactic acid reduces the production of CO_2, whereas an increase might have been expected owing to the inhibition of fixation of CO_2 by fluoride. That there was some fixation of CO_2 is indicated by the observed small decrease in succinic acid formation in the presence of fluoride.

An understanding of the mechanism of fixation of CO_2 may offer new avenues of investigation into the chemistry of photosynthesis [cf. Gaffron,[1] 1939]. Obviously the same type of mechanism could be common to the two processes. Preliminary results have shown that NaF affects the fermentation of galactose by *E. coli* much as it does the propionic acid fermentation; i.e. the production of CO_2 is increased and the succinic acid is decreased when fluoride is added. These results are interpreted as evidence of utilization of CO_2 with formation of succinic acid by colon bacteria. Fixation of CO_2 with formation of succinic acid

[1] Gaffron [1939] apparently misinterpreted the results of our previous investigations for he states that a net decrease in CO_2 has not been shown in the propionic acid fermentation.

may be a general biological reaction. Certainly activation of CO_2 by heterotrophic bacteria is a general phenomenon [cf. Wieringa, 1936; Woods, 1936; Barker, 1936; 1937 and Hess, 1938]. It should be emphasized, however, that there is one essential difference between the reactions referred to which involve fixation of CO_2. In the first place, there is the fixation as noted by the authors and Wieringa which involves reduction with a carbon-to-carbon linkage and, in the second place, that involving reduction with no linkage of carbon atoms. It is from an understanding of the carbon-to-carbon linkage that an elucidation of the reaction of assimilation may be expected.

Previously the authors have suggested that synthesis of citric acid by moulds may involve fixation of CO_2. In this connexion it is interesting that Johnson *et al.* [1939] have found that 0·01 *M* NaF inhibits formation of citric acid by *Aspergillus niger*. They suggest that inactivation of succinic dehydrogenase by NaF is the true cause of the inhibition. According to their proposals citric acid may be formed by a 4- and 2-carbon splitting of glucose or a breakdown to three 2-carbon compounds followed by synthesis with formation of succinic acid. The succinic acid is in turn activated by succinic dehydrogenase to combine with acetic acid to yield citric acid. In view of the results reported here, the NaF may be inhibiting a synthesis involving CO_2, and thus be preventing citric acid formation.[1] There is in reality no substantial proof of the often proposed 4- and 2-carbon cleavage of glucose [cf. Wood & Werkman, 1938].

Summary

The activity of cell suspensions of *Propionibacterium pentosaceum* (49 W) in fixing CO_2 depends on the medium used for growth. A glycerol-phosphate-yeast extract medium yields active cells. Inhibition of utilization of CO_2 by NaF causes a simultaneous decrease in the formation of succinic acid. This is further evidence that succinic acid may be formed by union of 1- and 3-carbon compounds. Apparently there is a second mechanism for the formation of succinic acid (particularly in glucose dissimilation) which is insensitive to fluoride. This fluoride-insensitive formation of succinic acid probably occurs by the condensation of acetic or pyruvic acid. CO_2 utilization is believed to be associated with a fluoride-sensitive phosphorylating mechanism. Attention is drawn to the possibility that fixation of CO_2 with formation of succinic acid may be a general biological phenomenon.

[1] An explanation of the inhibitive action of NaF on the utilization of CO_2 by propionic acid bacteria based on inactivation of succinic dehydrogenase is hardly acceptable because malonate and pyrophosphate do not affect fixation of CO_2 [cf. Wood & Werkman, 1940].

REFERENCES

Barker (1936). *Arch. Mikrobiol.* **7**, 404.
—— (1937). *Arch. Mikrobiol.* **8**, 415.
Friedemann & Graeser (1933). *J. biol. Chem.* **100**, 291.
Gaffron (1939). *Ann. Rev. Biochem.* **8**, 483.
Goodwin (1920). *J. Amer. chem. Soc.* **42**, 39.
Hess (1938). *Ann. Ferm.* **4**, 547.
Johnson, Knight & Walker (1939). *Biochem. J.* **33**, 357.
Munson & Walker (1906). *J. Amer. chem. Soc.* **28**, 663.

Osburn, Wood & Werkman (1933). *Ind. Engng Chem. Anal. Ed.* **5**, 247.
—— —— —— (1938). *Ind. Engng Chem. Anal. Ed.* **8**, 270.
Stiles, Peterson & Fred (1926). *J. Bact.* **12**, 427.
Werkman, Stone & Wood (1937). *Enzymologia*, **4**, 24.
Wieringa (1936). *Ant. Leeuwenhoek*, **3**, 89.
Wiggert & Werkman (1939). *Biochem. J.* **33**, 1061.
Wood & Werkman (1938). *Biochem. J.* **32**, 1262.
—— —— (1940). *Biochem. J.* **34**, 7.
Woods (1936). *Biochem. J.* **30**, 515.

22. GLYCOGEN

7. THE GLYCOGEN OF *HELIX POMATIA*

BY ERNEST BALDWIN AND DAVID JAMES BELL

From the Biochemical Laboratory, Cambridge

(*Received 12 December 1939*)

EARLY work by Claude Bernard, Hammarsten and others [see von Fürth, 1903; Biedermann, 1911 for references] left little doubt that in the metabolism of *Helix pomatia* the part played by glucose and glycogen is analogous to that which they play in the mammal. These earlier workers demonstrated the presence of glycogen in the hepatopancreas and in the tissues generally, its increase in feeding and its diminution in starvation. It is therefore interesting to find [Baldwin, 1938] that the metabolic behaviour of the hepatopancreas ("liver") of *Helix* is similar, in a general kind of way, to that of mammalian liver, an observation which might be regarded as giving some biochemical support to the view of Dorman [1928] that the hepatopancreas of molluscs and the liver of vertebrates are homologous. But the carbohydrate metabolism of *Helix* is peculiar in several respects. In common with certain other herbivorous gastropods such as *Pterocera* and *Strombus* [Yonge, 1932], *Helix* secretes a powerful extracellular cellulase which enables this snail to derive quantities of glucose from cellulose. This enzyme has been much studied by Karrer [e.g. Karrer & Illing, 1925]; its occurrence among Metazoa appears to be uncommon, for even among the highly specialized wood-eating insects it is by no means universally present [Mansour & Mansour-Bek, 1934]. Most remarkable, perhaps, is the presence in the organism of a laevorotatory, galactose-containing polysaccharide discovered by Hammarsten [1885] and named "tierisches Sinistrin" by him. This compound has been extensively studied from a predominantly biological viewpoint by May [1931; 1932; 1934], while more strictly chemical investigations of its structure have been carried out by Schlubach & Loop [1937] and by Baldwin & Bell [1938]. Both the glycogen and the galactogen of the snail presumably owe their origin largely to the products of the enzymic digestion of cellulose.

Biedermann [1911] gives references to early investigations in which glycogen was isolated from the hepatopancreas of *H. pomatia* and the finding of abnormally low values for its specific rotation. These were attributed at the time to admixture with the glycogen of the laevorotatory galactogen. May [1934] however found that galactogen is confined to the albumin gland, a conclusion which our present observations have confirmed; for, working with snails from which the albumin glands had previously been removed as a source of galactogen, we obtained typical preparations of glycogen which gave no evidence of containing or of being contaminated with galactose or galactogen. Our preparations gave a specific rotation of $+192°$ as compared with May's [1934] value of 196·6°. May [1934] observed also that while the albumin gland is rich in galactogen, it contains no glycogen and this too has been confirmed by our own work [Baldwin & Bell, 1938].

May [1934] showed that galactogen accumulates in the albumin gland during feeding and is transferred to the eggs when these are laid and that it must be

synthesized, in all probability, from glucose. If, during the winter sleep, the animals' reserves of glycogen become exhausted, it seems that the stores of galactogen can be drawn upon so that probably glucose (or glycogen) and galactose (or galactogen) are interconvertible in the organism. Baldwin [1938] has shown that the Q_{O_2} of sliced hepatopancreas tissue is increased by about one-third at most times of year by the addition of galactose or of galactogen though not, apparently, during the period which is normally spent in hibernation. Glucose and glycogen do not influence the Q_{O_2}.

In view of these various peculiarities and the somewhat confused state of the earlier literature it seemed to us desirable to isolate and study glycogen from *H. pomatia* with a view to finding out whether or not the unusual character of the carbohydrate metabolism of this form might be in some way reflected in peculiarities in the structure of its glycogen reserves. This seemed the more desirable in view of the fact [Bell, 1936, 1] that the glycogen of the liver of normal rabbits has a chain length of 12 units, while that from the livers of fasted rabbits fed with galactose by stomach tube has a chain length of 18 units. The only other molluscan glycogen of which the chain length has been determined is that from the whole tissues of *Mytilus edulis*, the edible mussel, and in this case a value of 18 units was found [Bell, 1936, 2]: other glycogens agree in having a chain length of only 12 units [cf. Bell, 1935, for fish liver glycogen, and 1937, for horse muscle glycogen: see also Haworth & Percival, 1932, and Haworth *et al.* 1937, who found values of 12 and 18 respectively for commercial samples of rabbit liver glycogen].

Experimental

Material used and isolation of glycogen. Our material consisted of the bodies of about 300 hibernating specimens of *H. pomatia* from which the albumin glands had been removed as a source of galactogen. The material was collected in alcohol, its total live weight, including shells, amounting to approx. ·5 kg. After expressing most of the alcohol the material was boiled with 30 % KOH for 5 hr., centrifuged to remove insoluble material, and precipitated with 2 vol. alcohol. The insoluble matter was again treated with hot KOH, centrifuged, precipitated with alcohol, and added to the main product. The combined crude products were dissolved in 50 % acetic acid, the solution was passed twice through the Sharples centrifuge to remove some insoluble matter, and the polysaccharide precipitated by the addition of 1 vol. alcohol. The product was then dissolved in 200 ml. very dilute acetic acid, centrifuged at high speed, and reprecipitated by the addition of 4 vol. glacial acetic acid. The precipitate was washed with 80 % acetic acid on the centrifuge, again dissolved in very dilute acetic acid, and once more precipitated from 80 % acetic acid. After dissolving in water and being precipitated by alcohol the product separated as a slightly brown powder which dissolved freely in water to give a very opalescent solution: yield 27 g. This, which corresponds very roughly to 0·5 % of the live weight of the original material, may be compared with the yields obtained by Bafurth and other workers [see von Fürth, 1903].

Purification and properties of the glycogen. 3 g. of the glycogen were reprecipitated four times from 80 % acetic acid, and finally from alcohol [cf. Bell & Young, 1934]. When thoroughly dried the product was a white powder which dissolved freely in water to give an opalescent solution. This gave a faint brown coloration with excess of iodine [cf. *Mytilus* glycogen, Bell, 1936, 2]: $[\alpha]_D^{20°} = +192°$ (water, $c=1$).

Acid hydrolysis. A 3·1 % solution in 2·5 % HCl was hydrolysed on a boiling water bath and gave the following polarimetric data, calculated in terms of polysaccharide:

t (min.) =	0	15	30	45	60	90	120	180	300
$[\alpha]_D$	= +192°	+143°	+106°	+83°	+72°	+62°	+59·5°	+58°	+57°

Expressed in terms of glucose the final $[\alpha]_D$ value is thus +51·7°, or 98 % of the theoretical value for an equilibrium mixture of α- and β-glucose. 20 ml. of the hydrolysate were neutralized with Ag_2CO_3, filtered, treated with H_2S and again filtered after treatment with charcoal, and finally evaporated to dryness. The product weighed 0·70 g. (theory 0·69 g.) and this was dissolved in hot 90 % alcohol and allowed to crystallize. Several crystalline fractions were obtained, totalling 0·64 g. (yield 93 %): M.P. 142–144° (uncorr.), mixed M.P. with an authentic sample of α-glucose showed no depression: $[\alpha]_D$ = +52·1° at equilibrium (water, c = 2). No sugar other than glucose was detected.

Acetylation. 22 g. of the crude glycogen were dissolved in 10 parts of water, precipitated by the addition of 3 vol. alcohol and at once collected on a Büchner funnel. After washing rapidly with alcohol and ether the now slightly moist precipitate was transferred to a flask and stirred mechanically with 400 ml. pyridine. Vigorous mechanical stirring was maintained during the addition of 350 ml. acetic anhydride and continued until a clear solution was obtained. After standing overnight at room temperature the solution was passed through a G 3 sintered Jena glass funnel into an excess of cold water. The precipitate was washed with cold water and dried; yield 35 g. (90 %). Found: $COCH_3$ = 44·3 %; $[\alpha]_D$ +160°, +161° (chloroform, c = 2). It may be pointed out that the specific rotation of this acetyl compound is about 8° lower than most of those in the literature.

Methylation. 33 g. of the triacetate were dissolved in 200 ml. acetone, warmed to 50° and treated with 400 ml. 30 % NaOH and 130 ml. dimethyl sulphate, these reagents being added in 10 equal portions at 10 min. intervals with violent mechanical stirring. After four such treatments, OMe = 39·0 %. After 8 methylations, OMe = 42·4 %. After each methylation the reaction mixture was heated till the acetone just began to boil and the aqueous layer then sucked off to leave the supernatant solution of the product in acetone. The acetone layer was then treated again with the methylating reagents. Finally, the product was obtained by boiling off the acetone, heating the whole on a boiling water bath for 40 min. to decompose dimethyl sulphate, and the precipitate was collected on a G 3 sintered Jena glass funnel and washed once with boiling water.

The crude methylated product was dissolved in chloroform, dried with anhydrous Na_2SO_4, filtered and evaporated to give a thin syrup from which a somewhat brown precipitate was separated by the addition of light petroleum. Further purification was effected by dissolving the material in acetone and adding light petroleum. After several such precipitations a slightly yellow powder was obtained with the following properties: OMe = 44·1 %: $[\alpha]_D$ +213° (chloroform, c = 1): soluble in acetone and chloroform, insoluble in hot water. Yield 16·8 g. (72 %). With excess of iodine this substance gave a faint brown coloration.

Hydrolysis of the methylated glycogen. 16·2 g. of the methylated glycogen were boiled for 8 hr. with 500 ml. methyl alcohol containing 1 % by weight of dry HCl [cf. Haworth *et al.* 1937]. The hot solution was then neutralized with $PbCO_3$, filtered when cold, and evaporated to dryness. The residue was extracted with chloroform and the resulting solution, now free from mineral matter, was

evaporated to dryness to give 17·6 g. of material. This was distilled at a pressure of 0·005 mm. Hg from a flask fitted with a vacuum-jacketed column, and we obtained the fractions described in Table 1.

Table 1

Fraction	Bath temp.	$n_D^{16°}$	$[\alpha]_D$ in water	% OMe	Weight, g.
I	101°	1·4425	+ 21·8	61·3	1·073
II	101–110°	1·4442	+ 69·0	58·0	0·312
III	110–130°	1·4552	+ 33·8	53·7	2·396
IV	130°	1·4581	+ 49·0	51·5	6·089
V	130–140°	1·4586	+120·7	51·5	2·966
VI	140–170°	1·4711	—	43·7	3·097
Residue	—	—	—	44·0	1·734

Employing the criteria of purity established by Hirst & Young [1938] for homogeneous fractions of 2:3:4:6-tetramethyl-methylglucosides and 2:3:6-trimethyl-methylglucosides we found that Fraction I consisted entirely of the former and Fractions IV and V of the latter. The distillation residue, a hard glass, consisted of a condensation product of 2:3:6-trimethyl glucose [cf. Haworth *et al.* 1937] and corresponded in amount to 1·85 g. of the methyl glucoside. By a method of approximate estimation based on Hirst & Young's [1938] procedure Fractions II and III were found to consist of

	II	III
Tetramethyl-methylglucoside	97·5%	13%
Trimethyl-methylglucoside	2·5%	87%

respectively. Fraction VI contains approximately 24·7% dimethyl-methyl-glucoside (calculated from methoxyl determinations), the remainder corresponding to trimethyl-methylglucoside. The amounts of the various fractions are tabulated in Table 2, and the total yield represents an approximately 90% recovery of the methylated glycogen. The chain length corresponds to 11–12 units, a value which differs from that of the only other specimen of molluscan glycogen which has been examined in this way [Bell, 1936, 2].

Table 2

Fraction	g. "Tetra"	g. "Tri"	g. "Di"
I	1·073	—	—
II	0·304	0·008	—
III	0·311	2·085	—
IV	—	6·089	—
V	—	2·966	—
VI	—	2·322	0·775
Residue	—	1·850	—
Totals	1·688	15·320	0·775
Total products		17·783	

Identification of the methylated sugars. Fractions I and II, hydrolysed by *N* HCl in the usual way, yielded 2:3:4:6-tetramethyl glucose, M.P. 88°, unchanged by admixture with an authentic sample.

Fractions III, IV and V, hydrolysed by *N* HCl in the usual way, yielded crystalline 2:3:6-trimethyl glucose, M.P. 102–105°, not depressed by admixture with an authentic sample. The syrupy mother liquors from these fractions were dissolved in methyl alcohol containing 1% by weight of dry HCl ($c=8·5$) and showed polarimetric behaviour characteristic of pure 2:3:6-trimethyl glucose; initial $[\alpha]_D+74°$, falling to a value of $-27°$ in 20 hr. at room temperature.

SUMMARY

1. Glycogen has been isolated from the bodies of specimens of *Helix pomatia* from which the albumin glands had been removed. No indications of the presence of any contaminating polysaccharide were found. This confirms the belief that galactogen is confined to the albumin gland.

2. The glycogen has been acetylated and methylated, and by the method of end-group assay the chain length has been estimated at 11–12 units, compared with the only other known value for molluscan glycogen (18 in the case of *Mytilus edulis* glycogen).

3. The glycogen of *Helix*, its acetate and the corresponding methylated compound, showed no significant differences from similar products obtained from other sources.

REFERENCES

Baldwin (1938). *Biochem. J.* **32**, 1225.
—— & Bell (1938). *J. chem. Soc.* p. 1461.
Bell (1935). *Biochem. J.* **29**, 2031.
—— (1936, 1). *Biochem. J.* **30**, 1612.
—— (1936, 2). *Biochem. J.* **30**, 2144.
—— (1937). *Biochem. J.* **31**, 1683.
—— & Young (1934). *Biochem. J.* **28**, 882.
Biedermann (1911). In Winterstein's *Handb. vergl. Physiol.* **2** (1), (Jena).
Dorman (1928). *J. Morph.* **45**, 537.
Hammarsten (1885). *Pflüg. Arch. ges. Physiol.* **36**, 373.
Haworth, Hirst & Isherwood (1937). *J. chem. Soc.* p. 577.
—— & Percival (1932). *J. chem. Soc.* p. 2277.
Hirst & Young (1938). *J. chem. Soc.* p. 1247.
Karrer & Illing (1925). *Helv. chim. Acta*, **8**, 245.
Mansour & Mansour-Bek (1934). *Biol. Rev.* **9**, 363.
May (1931). *Z. Biol.* **91**, 215.
—— (1932). *Z. Biol.* **92**, 321, 325.
—— (1934). *Z. Biol.* **95**, 277, 401, 606, 614.
Schlubach & Loop (1937). *Liebigs Ann.* **532**, 228.
von Fürth (1903). *Vergl. chem. Physiol. d. nied. Tiere* (Jena).
Yonge (1932). *Rep. Gt. Barrier Reef Exped. Brit. Mus.* (Nat. Hist.), **1**, 259.

23. THE METABOLISM OF OXALIC ACID IN THE ANIMAL BODY

By HAROLD HAYDEN BARBER and ERIC JAMES GALLIMORE

From the Sub-Department of Physiology, University College, Nottingham

(*Received 9 December 1939*)

The origin and fate of oxalic acid in the animal body have been studied almost exclusively by means of experiments involving the oral administration of the substance and the investigation of its metabolism in the body as a whole. Thus Mills [1885] showed that on a vegetable-free diet of flesh foods oxalate occurred in the urine. On a pure milk and sugar diet Lommel [1899] observed the excretion of oxalic acid and found that even during fasting oxalate had not disappeared from the urine on the 12th day. It was thus recognized that this substance could, in part at least, be of endogenous origin.

Proteins and sugars were early regarded as precursors of oxalate. Lommel [1899] found an increase in the urinary oxalate after the administration of gelatin. Klemperer & Tritschler [1902] stated that glycine and also creatine brought about an increased excretion of oxalate. More recently Pincussohn [1919] found an increased oxalate excretion after purine compounds had been administered either *per os* or intravenously to dogs. Yudeles *et al.* [1936] found that the oxalate of the blood was increased after the intravenous injection into dogs of small amounts of glycine. Bergrom [1935] found that the complete elimination of oxalate given by mouth required about 14 days and, since the excretion was quantitative, he suggested that this substance is not oxidized in the tissues.

Many more studies have been reported on similar lines with the object of demonstrating that the excreted oxalate is not exclusively of alimentary origin but is at least in part produced in the body, and also with the object of discovering the substances responsible for its production. On the other hand, little attention has been paid to the possibility of the study of oxalate formation *in vitro* either by surviving tissues or by intestinal flora. The present paper is an account of exploratory experiments in these latter directions.

Experimental

The determination of oxalate in blood and tissues

The method previously published for the estimation of oxalic acid in urine [Dodds & Gallimore, 1932] has been satisfactorily adapted for blood and tissues.

In the case of blood, 5 ml., treated with heparin, are added to 25 ml. water and 5 ml. 5*N* HCl are then added with shaking. After the addition of 2 g. phosphotungstic acid in 10 ml. water, followed by a further 5 ml. of 5*N* HCl, the whole is heated at 100° for 20 min. on the water bath. After cooling and allowing to stand for at least an hour, the precipitate is filtered off with suction on a Hirsch funnel and washed 3 times with 7 ml. of 5*N* HCl for each washing. The filtrate is collected in a Claisen flask and the contents then treated in the manner outlined for urine [Dodds & Gallimore, 1932]. Typical results showing the recovery of oxalate added to blood are given in Table 1.

Table 1

Sample	Oxalic acid added mg.	Oxalic acid found mg.	Added oxalic acid recovered mg.	Added oxalic acid recovered %
5 ml. distilled water	None	0·010	—	—
5 ml. distilled water	Heparin added	0·014	—	—
5 ml. defibrinated horse blood A	None	0·035	—	—
,,	None	0·027	—	—
,,	None	0·025	—	—
,,	1·0	0·88	0·85	85
,,	1·0	0·83	0·80	80
,,	1·0	0·88	0·85	85
,,	1·0	0·87	0·84	84
,,	0·1	0·113	0·084	84
,,	0·05	0·066	0·037	74
5 ml. defibrinated horse blood B	None	0·036	—	—
,,	Heparin added	0·031	—	—
5 ml. rabbit blood C	None	0·035	—	—
,,	0·1	0·117	0·082	82
5 ml. rabbit blood D	None	0·048	—	—
,,	0·03	0·076	0·028	93

All oxalic acid values are for anhydrous oxalic acid.

A number of such estimations of oxalate in the blood of normal adults showed the oxalic acid content to be between 0·4 and 0·6 mg. per 100 ml. of whole blood. In a patient with pronounced oxaluria the oxalic acid content of the blood was found to lie within this normal range. In the rabbit the content is slightly higher, varying from 0·4 to 0·8 mg. per 100 ml.

A similar procedure is applicable to tissues. The tissue, after being minced, is digested with a suitable amount of *N* HCl and phosphotungstic acid, the subsequent operations resembling those for blood.

Since the above assays are based on determinations involving an oxidation titration, it was thought desirable to check these values by a control experiment in which the oxidation titration was preceded by actual separation, weighing and microscopical identification of the calcium oxalate [Merz & Maugeri, 1931]. From 2·5 l. of defibrinated horse blood the proteins were removed with phosphotungstic acid and the oxalic acid was separated by distillation as ethyl oxalate; 16·2 mg. of an insoluble calcium salt were then obtained which under the microscope was identified as crystalline calcium oxalate. A potassium permanganate titration carried out on this precipitate was found to correspond with the calculated oxalic acid content.

Our findings for the oxalate content of blood support the statement of Thomsen [1935] that the methods of Merz & Maugeri [1931] and of Izumi [1933] give high and fictitious values for blood oxalate.

The effect of surviving tissues in vitro

Preliminary work showed that minced or sliced tissues did not destroy oxalic acid to any appreciable extent. The tissues studied were liver, kidney and spleen; the animals used included rats, guinea-pigs and rabbits. Sections as thin as practicable were cut from the fresh tissue with a razor and placed in saline or serum to which had been added the oxidizable substance. In some experiments the tissue was minced in a small machine such as is used for tumour implantations. In the majority of the experiments mentioned below controls

were performed alongside the actual digestion. In a typical experiment the tissue, weighed moist, was placed in a medium consisting of 25 ml. horse serum, 10 ml. normal saline and 0·2 g. creatine. The whole was maintained at 37° for 6 hr. whilst N_2 containing 5 % CO_2 was bubbled continuously through the fluid. The medium was then examined for oxalic acid.

The substrates included uric acid, glycine, ethylene glycol, glycerol, creatine and glucose. In some cases Ringer solution with a phosphate buffer to give a *p*H of either 6·0 or 8·0 was substituted for serum.

In none of these experiments was any formation of oxalic acid observed.

The action of intestinal organisms in vitro

At various times it has been suggested that the formation of oxalate is the result of simple fermentative changes occurring within the digestive tract [Mayer, 1902; Borjström, 1936].

It occurred to us that it might be possible to effect the formation of oxalic acid in a suitable medium *in vitro* by inoculation with faeces and incubation at body temperature. Contrary to expectations it was found that oxalate was not formed under conditions simulating those within the intestine, but that on the contrary any added oxalate was rapidly destroyed.

The medium used (Medium A) had the following composition (g./l.): NaCl, 9·00; KCl, 0·42; $CaCl_2.6H_2O$, 0·48; $NaHCO_3$, 0·50; K_2HPO_4, 0·25; $MgSO_4$, trace; glucose, 0·50; peptone, 2·00.

The solution, with or without the addition of known amounts of oxalate, was adjusted to the required *p*H by the addition of dilute NaOH and then sterilized in steam at atmospheric pressure for 3 days in succession. After inoculation with a suspension of human faeces equivalent to about 200 mg. in 100 ml. of medium A, incubation was carried out in 250 ml. conical flasks at 37°. Sterile control flasks of medium were incubated at the same time. The oxalate content of each flask was then determined.

Table 2. *Incubation of oxalate solutions at various* p*H values*

Vol. of medium A, 100 ml. Period of incubation, 7 days

Condition of medium	Initial *p*H	Oxalic acid added mg.	Oxalic acid found mg.
Sterile	7·5	102	102
+faeces	7·5	102	20
Sterile	7·0	102	108
+faeces	7·0	102	36
Sterile	6·5	102	108
+faeces	6·5	102	109

Table 3. *Incubation of oxalate solutions for various periods*

Vol. of medium A, 100 ml. Initial *p*H, 7·5

Condition of medium	Period of incubation days	Oxalic acid added mg.	Oxalic acid found mg.
Sterile	1	102	105
+faeces	1	102	102
,,	2	102	107
,,	3	102	18
,,	7	102	14
Sterile	7	102	102

Tables 2 and 3 illustrate the destruction of oxalate by faecal organisms at reactions of *p*H 7·0 and 7·5. Under these conditions the greater part of the oxalate is destroyed in 3 days.

Table 4. *Effect of oxygen*

Vol. of medium A, 100 ml. Initial *p*H, 7·5. Period of incubation, 7 days

Condition of medium	Oxalic acid added mg.	Oxalic acid found mg.
Aerobic, sterile	102	107
Aerobic, faecal inoculation	102	16
,, ,,	102	7
Anaerobic, sterile	102	108
Anaerobic, faecal inoculation	102	26
,, ,,	102	38

Table 4 indicates that the destruction of oxalate is independent of the presence of atmospheric oxygen and thus may occur under conditions resembling those which obtain within the intestine.

Table 5. *Effect of variations in the glucose and peptone contents*

Medium A without glucose and peptone; vol. 100 ml.; initial *p*H, 7·5; period of incubation, 7 days

Glucose and peptone added	Condition of medium	Oxalic acid added mg.	Oxalic acid found mg.
No peptone; 0·1 % glucose	Sterile	102	102
,, ,,	+faeces	102	102
0·2 % peptone; no glucose	Sterile	102	100
,, ,,	+faeces	102	80
0·2 % peptone; 0·05 % glucose	Sterile	102	102
,, ,,	+faeces	102	14
0·2 % peptone; 0·1 % glucose	Sterile	102	104
,, ,,	+faeces	102	12
0·2 % peptone; 0·3 % glucose	Sterile	102	105
,, ,,	+faeces	102	102

In Table 5 the negative result obtained in the absence of peptone is possibly accounted for by the inability of the organisms to function in a peptone-free medium. The results without and with various amounts of glucose suggest that this substance is concerned in the oxalate oxidation. The absence of oxalate destruction in the higher concentrations of glucose is possibly explained by the observed rapid change of the reaction of the medium during incubation to an acidity which would inhibit this action of the organism.

Table 6. *Effect of partial sterilization of the faecal inoculum; effect of toluene*

Vol. of medium A, 100 ml. Initial *p*H, 7·5. Period of incubation, 7 days

Condition of medium	Oxalic acid added mg.	Oxalic acid found mg.
+faeces	102	32
+faeces. Suspension previously heated at 80° for 20 min.	102	105
,, ,,	102	102
+faeces	25	2·5
+faeces and 0·5 % toluene	25	23

The absence of oxalate destruction by a faecal inoculum previously heated at 80° indicates that the effect is dependent on the activity of non-sporing organisms.

The negative result in the presence of toluene eliminates the possibility of oxalate destruction by enzymes introduced in the faecal inoculum.

According to the foregoing results destruction of oxalic acid occurs only at a *p*H above 7·0. A few experiments were made to determine the effect of a faecal inoculation at an acidity nearer to that of the stomach. Under these conditions oxalic acid formation was found to take place.

The medium employed was the same as medium A but with the glucose content raised to 2%.

A volume of 500 ml. of this medium was adjusted to *p*H 2·4. After sterilization separate portions, each of 100 ml., were inoculated, (*a*) with a suspension of faeces, (*b*) with a scraping from a sound tooth, whilst a third portion without treatment acted as a control. After incubation for 10 days the control medium gave no oxalic acid, whilst the calcium oxalate isolated from the faecal inoculation corresponded to 4·2 mg. and that from the tooth deposit inoculation to 3·1 mg. of oxalic acid.

Summary

1. A method for the estimation of oxalic acid in blood is described. The oxalic acid content of normal blood was found to lie between 0·4 and 0·6 mg. per 100 ml. whole blood.

2. Tissues do not form or destroy oxalic acid under the conditions described.

3. At a *p*H value of 7·0 or higher a suspension of faeces inoculated into a nutrient solution containing oxalate causes the destruction of oxalate on incubation at 37°. This was observed under both aerobic and anaerobic conditions. The action is probably due to a non-sporing organism.

4. At a *p*H value of 2·4 a similar inoculation with somewhat longer incubation at 37° was found to give rise to oxalic acid.

REFERENCES

Bergrom (1935). *Biochem. Z.* **281**, 377.
Borjström (1936). *Skand. Arch. Physiol.* **73**, 63.
Dodds & Gallimore (1932). *Biochem. J.* **26**, 1242.
Izumi (1933). *Jap. J. med. Sci.* II, *Biochem.* **2**, 195.
Klemperer & Tritschler (1902). *Z. klin. Med.* **44**, 387.
Lommel (1899). *Dtsch. Arch. klin. Med.* **63**, 599.
Mayer (1902). *Z. klin. Med.* **47**, 112.
Merz & Maugeri (1931). *Hoppe-Seyl. Z.* **29**, 435.
Mills (1885). *Virchows Arch.* **99**, 129.
Pincussohn (1919). *Biochem. Z.* **99**, 276.
Thomsen (1935). *Hoppe-Seyl. Z.* **237**, 199.
Yudeles, Genkin & Korshunova (1936). *Arch. Sci. biol., U.S.S.R.* **41**, No. 1, 117.

24. THE HEMICELLULOSES OF THE WOOD OF ENGLISH OAK

5. THE STRUCTURE OF HEMICELLULOSE B

By MARGARET HELENA O'DWYER

From the Section of Chemistry, Forest Products Research Laboratory, Princes Risborough, Aylesbury, Bucks

(*Received 21 December 1939*)

Whereas hemicellulose A is obtained on adding excess acetic acid to a cold 4% NaOH extract of wood, two methods are available for preparing hemicellulose B. In the first place the latter is precipitated on adding 2 volumes of alcohol to the mother liquors from which hemicellulose A has previously been removed [O'Dwyer, 1926], and secondly it has been shown [O'Dwyer, 1931] that a certain amount is obtained as a flocculent precipitate on adding an excess of alcohol to a 0·2% NaOH extract of wood. In a previous communication [O'Dwyer, 1931] the effect of various methods of drying the parent wood substance on the yield and composition of the inherent hemicellulose A was reported. Hemicellulose B has since been prepared from the same starting materials by the methods indicated above and the experimental data now presented concern the structure of this substance as well as its relationship to hemicellulose A.

Experimental

Purification and properties of hemicellulose B

The crude product obtained from sawdust (previously extracted with water at 100°) by extraction with 0·2% NaOH for 48 hr. at room temperature was much darker in colour than that obtained from the hemicellulose A mother liquors. Purification was effected by repeated solution in water and precipitation with alcohol and the final product was dehydrated in the usual way with alcohol and ether. No precipitation occurred with acetone [cf. Norris & Preece, 1930]. The material obtained from each wood sample by both methods of extraction was combined. Hemicellulose B as thus prepared is a light fawn to almost white impalpable powder and is soluble in water. It does not reduce Fehling's solution and with naphthoresorcinol gives a positive reaction for uronic acids and their anhydrides. As indicated in Tables 1 and 2 the yields of hemicellulose B obtainable from oak wood are in all cases smaller than those of hemicellulose A [O'Dwyer, 1931]. It was found that all the preparations of hemicellulose B from sapwood gave a violet coloration with iodine but no change in colour was observed on adding iodine to the heartwood products.

The various preparations of hemicellulose B have been analysed (Tables 1 and 2) according to the methods previously described [O'Dwyer, 1934; 1939] and the data are so expressed as to be directly comparable with those given previously for hemicellulose A [O'Dwyer, 1939].

Table 1. *Analysis of hemicellulose B from oak sapwood*

Source of hemicellulose	Yield as % of oven-dry wt. of starting material	Specific rotation (in water $c=0{\cdot}8$)	Ash %	% on ash-free basis of: Mono-methyl-uronic anhydride	Xylan	Methoxyl %	% organic matter accounted for
Green wood	1·5	+25·5°	3·64	16·81	59·53	2·02	76·34
Air-dried wood	2·0	+20·0°	3·27	17·11	64·33	2·0	81·44
Kiln-dried wood (maximum kiln temperature 40·6°)	2·5	+44·6°	3·2	15·39	55·30	1·98	70·69
Kiln-dried wood (maximum kiln temperature 54·4°)	3·6	+69·6°	1·89	12·03	48·82	1·90	60·85
Oven-dried wood	3·5	+63·6°	3·9	13·88	52·23	1·95	66·11

Table 2. *Analysis of hemicellulose B from oak heartwood*

Source of hemicellulose	Yield as % of oven-dry wt. of starting material	Specific rotation (in water $c=0{\cdot}4$)	Ash %	% on ash-free basis of: Mono-methyl-uronic anhydride	Xylan	Methoxyl %	% organic matter accounted for
Green wood	1·2	−52·3°	1·75	16·87	82·03	2·24	98·90
Air-dried wood	1·3	−52·8°	2·40	16·42	82·0	2·30	98·42
Kiln-dried wood (maximum kiln temperature 40·6°)	1·3	−53·7°	1·56	17·04	80·9	2·18	97·94
Kiln-dried wood (maximum kiln temperature 54·4°)	2·0	−53·2°	2·10	17·15	81·2	2·10	98·35
Oven-dried wood	1·8	−53·1°	2·98	17·20	79·5	2·09	96·70

Action of takadiastase on hemicellulose

It is observed from Table 1 that after adding the percentage of xylan to that of monomethyluronic anhydride a large percentage of sapwood hemicellulose B is unaccounted for. In contradistinction to the other hemicelluloses examined by the author, sapwood hemicellulose B was found to have a positive rotation and this, coupled with the observation that it gave a violet coloration with iodine, suggested that it might contain anhydroglucose units. Separate samples of all the hemicellulose B preparations (Tables 1 and 2) were digested with takadiastase for 48 hr. under the conditions already described for hemicellulose A [O'Dwyer, 1937; 1939]. In each case the hydrolysate reduced Fehling's solution and the addition of an excess of alcohol resulted in the precipitation of a polysaccharide of $[\alpha]_D^{20°} = -51{\cdot}2°$. The only sugar produced during the enzymic hydrolysis of the sapwood product was *d*-glucose. Found for the osazone M.P. 224° and for the *p*-bromophenylhydrazone M.P. 148° [Fischer & Paulus, 1935]. *d*-Xylose was the only sugar formed from the heartwood product and it was identified through cadmium bromide-cadmium xylonate (boat-shaped crystals) and the osazone. Found for the latter M.P. 163° [Fischer & Paulus, 1935]. After removal of as much as possible of the water-soluble polysaccharide

the glucose remaining after the enzymic hydrolysis of each of the sapwood products was determined titrimetrically [Lane & Eynon, 1934] and expressed as a percentage of the appropriate starting material (Table 2). The yields of xylose from the heartwood products were small and when expressed as glucose corresponded on the average to 3·2 % of original hemicellulose.

Table 3. *% of anhydroglucose units in oak sapwood hemicellulose B as determined by titration after enzymic hydrolysis*

Source of hemicellulose	Wt. taken (oven-dry) g.	Sugars as glucose in hydrolysate g.	Sugars as % anhydroglucose in original hemicellulose
Green wood	1·2192	0·3325	24·6
Air-dried wood	1·2813	0·2887	20·3
Kiln-dried wood (maximum temperature 40·6°)	2·0312	0·4188	32·8
Kiln-dried wood (maximum temperature 54°)	2·0399	0·8644	37·2
Oven-dried wood	2·1000	0·8100	34·8

Composition of the water-soluble polysaccharide of $[\alpha]_D^{20^\circ} = -51{\cdot}2^\circ$

The yields of the soluble polysaccharide precipitated by alcohol after the enzymic hydrolyses referred to above were small and in no sense quantitative, partly owing to difficulties in collection, but largely it is believed owing to incomplete hydrolysis of the starting material. A composite sample (4·66 g.) representative of all the hemicellulose preparations (Tables 1 and 2) was completely hydrolysed by heating with 1 % H_2SO_4 at 100° for 3 hr. After neutralizing with $BaCO_3$ a barium salt (1·35 g.) of $[\alpha]_D^{21^\circ} = +70^\circ$ ($c = 0{\cdot}4$ in water) and crystalline xylose (2·23 g.) were isolated. Found for the Ba salt, uronic anhydride 42·93 %, Ba 16·9 %, MeO 7·01 % and free aldehyde 6·9 %. Furfuraldehyde phloroglucide representing 41 % of xylose was also obtained from this substance as calculated from Kröber's tables.

Discussion

The data in Table 3 indicate that the anhydroglucose units which have been shown to be present in sapwood hemicellulose B account for its positive specific rotation. Variations in specific rotation which will be discussed later are closely shadowed by corresponding variations in the content of anhydroglucose residues. By adding the appropriate percentages of anhydroglucose (Table 3) to the percentage of organic matter accounted for in each of the preparations in Table 1 the whole of the organic matter in the original hemicellulose is accounted for. Heartwood hemicellulose B contains no anhydroglucose residues but the two hemicelluloses nevertheless have certain constitutional features in common. Both contain xylose and uronic acid residues and, on enzymic hydrolysis, both yield a polysaccharide of $[\alpha]_D^{20^\circ} = -51{\cdot}2^\circ$.

The specific rotation and analytical data for this polysaccharide correspond so closely to those of the polysaccharide already isolated from hemicellulose A [O'Dwyer, 1934; 1939] that there is little room for doubt that all of the oak hemicelluloses so far examined contain a recurring structural unit consisting of 6 anhydroxylose units and 1 monomethylhexuronic anhydride unit.

Reference to Tables 1 and 2 gives some insight into the effects of the various methods of drying the parent wood substance on the composition of hemicellulose B. In the case of the sapwood product it is observed (Table 1) that,

although drying of the parent wood at temperatures above room temperature results in slightly increased yields, heat has induced some degradation which is probably hydrolytic in character. Decreases are recorded in all of the components except the anhydroglucose residues (Table 3) and here substantial apparent increases have taken place. Heartwood hemicellulose B is remarkably constant in composition irrespective of its source and it would therefore appear to be much more resistant to decomposition by heat than the sapwood product.

Summary

1. Oak sapwood hemicellulose B resembles hemicellulose A from the same source in that it contains anhydroglucose units. Heartwood hemicellulose B, like heartwood hemicellulose A, contains no anhydroglucose units.

2. All of the oak hemicelluloses so far examined give rise to two common fission products on hydrolysis with takadiastase under standardized conditions, namely xylose and a water-soluble polysaccharide of $[\alpha]_D^{20^\circ} = -51{\cdot}2^\circ$ the molecule of which is composed of 1 monomethylhexuronic acid residue and 6 xylose residues.

The work described above was carried out as part of the programme of the Forest Products Research Board and is published by permission of the Department of Scientific and Industrial Research. The author's thanks are due to Mr W. G. Campbell, Officer in Charge, Chemistry Section, for many helpful suggestions.

REFERENCES

Fischer & Paulus (1935). *Arch. Pharm. Berl.* **273**, 83.

Lane & Eynon (1934). Determination of reducing sugars by Fehling's solution with methylene blue as indicator. London: Norman Rodger.

Norris & Preece (1930). *Biochem. J.* **24**, 59.

O'Dwyer (1926). *Biochem. J.* **20**, 656.

—— (1931). *Biochem. J.* **25**, 2017.

—— (1934). *Biochem. J.* **28**, 2116.

—— (1937). *Biochem. J.* **31**, 254.

—— (1939). *Biochem. J.* **33**, 713.

25. SOME FACTORS INFLUENCING THE ANTICOAGULANT ACTION OF HEPARIN

By LOUIS BARKER JAQUES and ROBERT ALEXANDER MUSTARD

From the Department of Physiology, University of Toronto, Toronto, Canada

(*Received 18 December 1939*)

It has been generally assumed that the anticoagulant action of heparin is due to direct combination with prothrombin, thrombin, or kinase. The chief experimental facts on this point have been confirmed by Mustard & Jaques [1937] and may be summarized as follows. When citrated plasma and thrombin are used to test the action of heparin, the heparin acts as an antithrombin [Howell, 1925]. When kinase is added, it neutralizes the action of heparin. It is thus possible to "titrate" heparin against kinase [Fischer & Astrup, 1935]. If the prothrombin is removed from the plasma with alumina gel, the heparin is still active but the corresponding action of kinase is largely abolished [Quick, 1936]. Heparin thus acts as an antikinase indirectly. Its anticoagulant action is that of an antithrombin. It has been frequently suggested (originally by Howell) that heparin also acts as an antiprothrombin, and recently Brinkhous *et al.* [1939] have obtained direct evidence of such an antiprothrombin action. In this paper, however, only the antithrombin action of heparin will be considered. The question of the anticoagulant action of heparin is complicated by the influence of various factors outside the known clotting system. Mellanby [1934] concluded that the presence of neutral salts was necessary for the action of heparin: Howell & Holt [1918] concluded that the serum proteins, particularly the euglobulin fraction, were necessary. We have not found any evidence that salt is necessary for the action of heparin but have confirmed the work of Howell & Holt and of Quick, that the presence of serum protein is essential

Methods

The activity of any factor in the clotting system can be measured either by direct determination of the resulting clotting time or by determination of the dilution necessary to give a fixed clotting time. In either case an arbitrary standard solution must always be used for comparison. The clotting time method has the disadvantage that the clotting time is not a simple function of the heparin concentration [Barratt, 1937]. This renders difficult the interpretation of data on the effect of other factors on the action of heparin. The dilution method with a fixed clotting time is used in the assay of heparin by Charles & Scott [1933] and this can be adapted to various purposes. Its chief disadvantage is the great number of tests which it is necessary to conduct in order to obtain quantitative results with any degree of accuracy. A third method which has definite but limited advantages is to determine the rate of clotting by measurements of turbidity, viscosity, or fibrin nitrogen [*vide* Jaques, 1938]. With any method considerable care must be taken when comparing two different anticoagulant substances since Astrup [1938] has found quite different relationships for different anticoagulants.

We have used the method which appeared to be the most suitable for the experiment planned. Constant temperature water baths were used. The experiments were carried out in 2 ml. coagulation tubes with a total volume of 1·0 ml. of the clotting mixture. The clotting time was determined at 37·5°. The dilution method was carried out at 25° with a fixed clotting time of 2 hr.

Materials

Fibrinogen and thrombin were prepared by the methods of Florkin and Mellanby respectively as previously described [Jaques, 1938]. The heparin used was supplied by the Connaught Laboratories. In the early experiments only the 5 unit per mg. material was available: later, as the 15, 60 and 100 unit materials were prepared, these were used. There was no apparent difference in these preparations as regards the experiments described here. The unit used is that of the Connaught Laboratories, Toronto, and has been found to be equivalent to the potency of 1/100 mg. of the crystalline barium salt of heparin prepared by Charles & Scott [1936].

The effect of salt on the action of heparin

Mellanby [1934] has emphasized the effect of salt concentration on the action of heparin. He found that dialysis of plasma resulted in a loss of heparin activity and that this was restored by the addition of NaCl. Hence, he concluded that the presence of neutral salt was necessary for the action of heparin. While under certain limited conditions this appears to be the case, we have found that when a more extensive study of the system is made, the effect of NaCl is to depress the activity of heparin rather than to increase it.

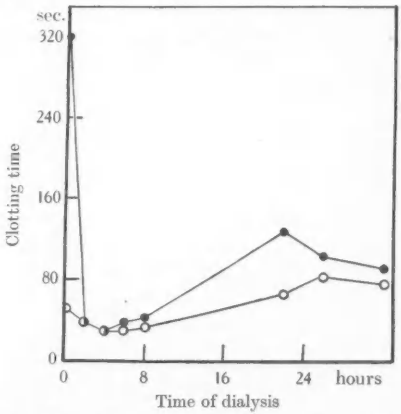


Fig. 1. Effect of dialysis on the antithrombin action of heparin. o—o Clotting time with 0·5 ml. of thrombin. •—• Clotting time with 0·5 ml. of thrombin +0·1 unit of heparin.

Citrated horse plasma was dialysed against distilled water and 5 ml. samples were removed from time to time and filtered. 0·5 ml. of a standard thrombin solution was added to 0·5 ml. of the sample and the clotting time determined. The clotting time was also determined with a second 0·5 ml. of the sample to which was added the standard thrombin +0·1 unit of heparin. The results are shown in Fig. 1. The clotting time of the plasma with thrombin alone decreased

to a minimum, due to removal of salts, and then gradually increased. The effect on the action of heparin was different. In the early stages of the dialysis the clotting time with heparin decreased until it was the same as the control, as observed by Mellanby. With prolonged dialysis, however, the clotting time with heparin increased over that of the control, i.e. there appeared to be a point during dialysis where the heparin had least activity. In further experiments plasma was dialysed for 18 hr. and then 20 % NaCl was added to samples of it to give a series of plasmas with known salt concentrations. Varying amounts of heparin were then added to 0·5 ml. of the samples and the clotting times with a constant amount of thrombin determined. The results are shown in Fig. 2. It can be observed (Fig. 2 (1)) that for each heparin concentration, as the salt

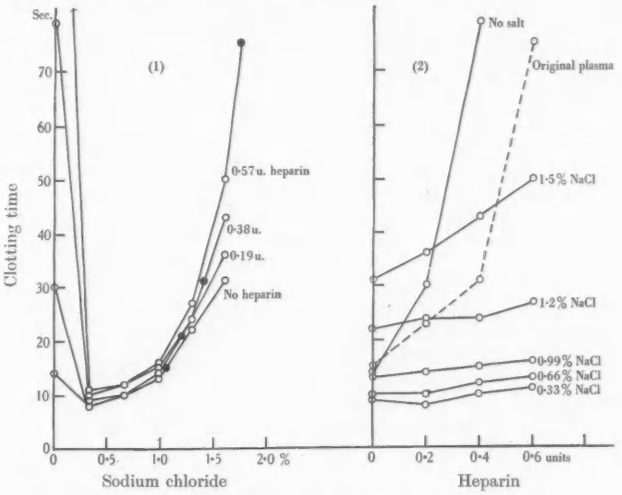


Fig. 2. Effect of sodium chloride concentration on heparin activity.

concentration increased, the clotting time decreased to a minimum and then increased. The value of the salt concentration for this minimum for the various curves was approximately the same. The average figure of a number of experiments was 0·5 %. To make it possible to examine the data more fully, they have been replotted in Fig. 2 (2). Clotting time is plotted against heparin concentration and a series of curves are obtained, the slope of any one of which gives a measure of the heparin activity for that particular salt concentration. It can be seen that on dialysis the heparin activity has been increased. With 0·4 unit of heparin, for example, the clotting time has been increased from 30 to 80 sec. and this appears to be a direct effect on heparin as the clotting time with thrombin alone has not changed. When NaCl was added to the dialysed plasma, however, the action of heparin was almost completely neutralized. This is in contrast to the action of NaCl on thrombin alone (Fig. 2 (1)). NaCl added to dialysed plasma first increases the activity of thrombin (lowers the clotting time) and then decreases it, i.e. traces of NaCl have a coagulant action whilst larger amounts have an anticoagulant action. At all salt concentrations, however, the NaCl tends to neutralize the action of heparin. Only in the case of 1·5 % NaCl does the heparin

show any activity and this may be wholly due to the fact that the thrombin activity has by then been greatly reduced (clotting time for zero heparin has been doubled). None of the heparin curves with added salt show any resemblance to those obtained with either the original plasma or the dialysed plasma. It should be observed that all the above results were obtained with one sample of dialysed plasma. The experiment has been repeated a number of times with the same results, using plasmas from different horses. It appears from these results that NaCl decreases the activity of heparin. This can only be observed at very low concentrations of heparin and salt (i.e. highly dialysed plasma). At higher concentrations the clotting time increases so rapidly with an increase in the concentration of either that it is not possible to detect the much smaller effect on the activity of the heparin due to the presence of the salt. Thus, with undialysed plasma or plasma which has been dialysed only a short time, the addition of more NaCl to that already present causes a marked increase in clotting time and disguises the direct effect of the salt on the heparin.

The effect of plasma proteins on the action of heparin

Howell concluded that heparin did not act as a simple antithrombin since it only acted as an antithrombin in plasma or blood but was inactive with fibrinogen solutions. This was confirmed in an experiment to test the purity of the fibrinogen. A fibrinogen solution and a sample of the original horse plasma were used, and increasing amounts of heparin were titrated against increasing amounts of thrombin, a fixed clotting time of 2 hr. at 25° being the end-point. Analyses for fibrin N, chloride and phosphate and *p*H determinations by the glass electrode showed the fibrinogen solution and the plasma to be identical in regard to these factors. It was observed, however, that while heparin is highly potent when plasma is used for testing its potency, it is almost completely inactive when a solution of fibrinogen is substituted for the plasma. Addition of dialysed plasma to the fibrinogen solution restores the activity of heparin.

The nature of the plasma protein factor involved was studied by a fractional precipitation of the plasma with $(NH_4)_2SO_4$. 25 ml. portions of plasma were precipitated with $(NH_4)_2SO_4$, centrifuged and the precipitates dissolved in 25 ml. of physiological saline. Their potency was then tested by determining the amount it was necessary to add to 1 unit of heparin in order to keep a clotting mixture fluid for 2 hr.—0·5 ml. of fibrinogen solution + 0·02 ml. of thrombin + 1·0 unit of heparin + saline to 1 ml. were taken for the test. Without plasma this mixture clotted in 10 min. The results are shown in Table 1. The protein

Table 1

Fraction	ml. required for heparin end-point	Potency as % of plasma	Antithrombin potency—clotting time min.
Plasma	0·005	—	25
Precipitated by $\frac{1}{3}$ sat. $(NH_4)_2SO_4$	>0·50	0	10
Precipitated by $\frac{1}{2}$ sat. $(NH_4)_2SO_4$	0·06	7	10
Full sat. $(NH_4)_2SO_4$	0·005	100	25
Crystalline serum albumin	>0·5	0	10

The serum albumin was crystallized from 250 ml. horse plasma and dissolved in 25 ml. of saline. The solution contained 3·82 mg. protein N per ml.

factor necessary for heparin activity is found in the albumin fraction, but is not associated with crystalline albumin. Quick [1938] has identified the protein factor with the normal antithrombin of plasma. The antithrombin potencies of

the various fractions are also shown in Table 1. The thrombin + 0·1 ml. of the protein solution were mixed and allowed to stand in the ice-box overnight. 0·5 ml. of fibrinogen was then added and the time taken to clot was noted. It can be observed that the antithrombin potency is also found in the albumin fraction but not in the solution of crystalline serum albumin.

Discussion

The results reported here indicate that the presence of NaCl decreases the anticoagulant action of heparin. It is evident, however, that usually this action is overshadowed by the direct anticoagulant action of sodium chloride as observed by Mellanby. It is also shown that heparin only acts as an antithrombin in the presence of plasma protein. There are two possible explanations for this fact. Either heparin is the anticoagulant but is only active when combined with protein, or the protein is the anticoagulant and heparin acts by combining with it and enhancing its activity. The latter hypothesis has been advanced by Quick. Quick suggests that heparin acts by combining with the normal antithrombin of serum. This so increases the affinity of the normal antithrombin for thrombin that it competes with fibrinogen for the thrombin, thus becoming an anticoagulant. The results reported here are in agreement with those of Quick although the low activity of the plasma antithrombin renders difficult its assay and an adequate testing of the hypothesis. Quick has suggested the term "heparin antithrombin" for the combined anticoagulant. Further evidence in favour of Quick's hypothesis is the work of Fischer on the union of heparin with proteins. He found that, while heparin combines with a large number of proteins under suitable conditions (on the acid side of the isoelectric point of the protein), the resulting heparin-protein compound is inactive provided that it is stable at the *p*H of the assay. We have obtained results similar to those of Fischer.

Howell, in his original paper on the subject, showed that the protein was destroyed by heating to 70°. Howell thought it was a euglobin, Quick thinks that it is the serum albumin. From the results reported, it is seen that the substance is found in the albumin fraction of plasma but is not crystalline albumin. Provided that the reaction of heparin with this protein is similar to that of heparin with other proteins, as described by Fischer, it is probable that its isoelectric point is more alkaline than the *p*H of plasma.

Little is known of the resulting heparin antithrombin. Howell concluded that heparin antithrombin is different from serum antithrombin as the latter is destroyed by heat and as no metathrombin develops in heparinized serum. Schmitz & Kühl [1935], however, observed destruction of heparin antithrombin in serum at 56°. We have found that it is precipitated by saturated Na_2SO_4 and $(NH_4)_2SO_4$. The formation of heparin antithrombin is not instantaneous but involves a considerable period of time after the addition of the heparin to the blood. This was observed by Quick who found that the apparent potency of heparin in blood *in vitro* increased on standing. The same phenomenon can be observed *in vivo*.

Summary

1. Prolonged dialysis of plasma has little effect on the activity of heparin. Addition of sodium chloride to this plasma decreases the activity of the heparin.

2. The results of Howell which indicated that heparin is only active in the presence of a protein in plasma are confirmed. The protein is found in the albumin fraction but is not crystalline serum albumin.

3. These results are in agreement with Quick's view that heparin acts by combining with and enhancing the activity of the normal plasma antithrombin.

REFERENCES

Astrup (1938). *Enzymologia*, **2**, 377.
Barratt (1937). *J. Physiol.* **90**, 377.
Brinkhous, Smith, Warner & Seegers (1939). *Amer. J. Physiol.* **125**, 683.
Charles & Scott (1933). *J. biol. Chem.* **102**, 425.
—— —— (1936). *Biochem. J.* **30**, 1927.
Fischer & Astrup (1935). *Biochem. Z.* **278**, 326.
Howell (1925). *Amer. J. Physiol.* **71**, 553.
—— & Holt (1918). *Amer. J. Physiol.* **47**, 328.
Jaques (1938). *Biochem. J.* **32**, 1181
Mellanby (1934). *Proc. Roy. Soc.* B, **116**, 1.
Mustard & Jaques (1937). *Trans. Roy. Soc. Can.* **28**, Appendix B, clvi.
Quick (1936). *Amer. J. Physiol.* **115**, 317.
—— (1938). *Amer. J. Physiol.* **123**, 712.
Schmitz & Kühl (1935). *Hoppe-Seyl. Z.* **234**, 212.

LINDA HALL LIBRARY
KANSAS CITY, MO.

26. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

64. EMODIC ACID (4:5:7-TRIHYDROXYANTHRAQUINONE-2-CARBOXYLIC ACID) AND ω-HYDROXYEMODIN (4:5:7-TRIHYDROXY-2-(HYDROXYMETHYL)-ANTHRAQUINONE), METABOLIC PRODUCTS OF A STRAIN OF *PENICILLIUM CYCLOPIUM* WESTLING

BY WINSTON KENNAY ANSLOW, JOHN BREEN AND HAROLD RAISTRICK

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London

(*Received 21 December 1939*)

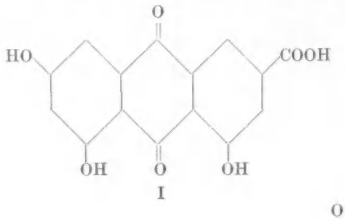
A NUMBER of polyhydroxyanthraquinones, all of which are derivatives of 2-methylanthraquinone, have been reported from these laboratories as metabolic products of different species of moulds. They are: helminthosporin, 4:5:8-trihydroxy-2-methylanthraquinone, from *Helminthosporium gramineum* Rabenhorst [Charles *et al.* 1933]; cynodontin, 1:4:5:8-tetrahydroxy-2-methylanthraquinone, from *H. cynodontis* Marignoni [Raistrick *et al.* 1933]; catenarin, a 1:5:8-trihydroxy-β-(hydroxymethyl)-anthraquinone, from *H. catenarium* Drechsler [Raistrick *et al.* 1934]; tritisporin, a 1:3:5:8-tetrahydroxy-β-(hydroxymethyl)-anthraquinone from *H. tritici-vulgaris* Nisikado [Raistrick *et al.* 1934]; physcion, 4:5-dihydroxy-7-methoxy-2-methylanthraquinone [Raistrick *et al.* 1937] and erythroglaucin, a monomethyl ether of a tetrahydroxymethylanthraquinone of at present undetermined molecular constitution, both of which were isolated from a large number of species in the *Aspergillus glaucus* series [Ashley *et al.* 1939]. The purpose of the present communication is to describe an investigation of the colouring matters present in the mycelium of a strain of *Penicillium cyclopium* Westling from which have been isolated two further derivatives of 2-methylanthraquinone, i.e. emodic acid, 4:5:7-trihydroxyanthraquinone-2-carboxylic acid and the hitherto undescribed *ω-hydroxyemodin, 4:5:7-trihydroxy-2-(hydroxymethyl)-anthraquinone*.

The strain of *P. cyclopium* used in this investigation (L.S.H.T.M. Cat. No. P 214), while otherwise morphologically indistinguishable from three other strains in our collection, differs from them in giving, under certain well defined conditions, a bright orange reverse. This colouring matter readily dissolves on treating the mould with cold dilute sodium carbonate solution to give an intensely crimson extract. Methods are described for the isolation of the colouring matter and for its fractionation into its constituents, emodic acid and ω-hydroxyemodin, which are present in approximately equal proportions and amount together to about 1·5% of the dry weight of the mould.

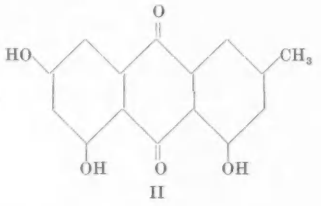
Emodic acid (I), $C_{15}H_8O_7$, M.P. 363–365°, forms orange needles which are readily soluble in cold dilute sodium bicarbonate sclution. It gives a *monomethyl ester* and a triacetyl derivative which itself gives a monomethyl ester. The

identity of the mould emodic acid was placed beyond all doubt by direct comparison of the acid, its *methyl ester*, triacetate and *triacetyl methyl ester* with the corresponding derivatives of synthetic emodic acid prepared by the oxidation of the triacetate of *Frangula*-emodin with chromic acid in acetic anhydride-glacial acetic acid solution according to the method of Fischer & Gross [1911]. The constitution of emodic acid (I) as 4:5:7-trihydroxyanthraquinone-2-carboxylic acid follows from that of *Frangula*-emodin (II), 4:5:7-trihydroxy-2-methylanthraquinone, which has been settled by synthesis by Eder & Widmer [1923], Eder & Hauser [1925] and Jacobson & Adams [1924]. Although emodic acid has been prepared from *Frangula*-emodin by purely chemical means by a number of workers [Fischer & Gross, 1911; Eder & Hauser, 1925; Asahina & Fuzikawa, 1935], it has not been reported previously, so far as we are aware, from any natural source.

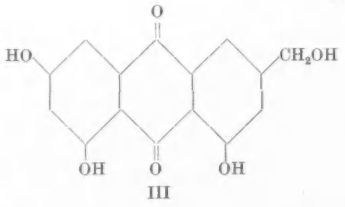
ω-Hydroxyemodin (III), $C_{15}H_{10}O_6$, forms dull orange needles, M.P. 288°. It is insoluble in sodium bicarbonate solution but, as is usual with hydroxyanthraquinones having a β-hydroxyl group, it readily dissolves in cold dilute sodium carbonate solution. It forms a *tetraacetate*, M.P. 190–191°, which on oxidation with chromic acid in acetic anhydride-glacial acetic acid solution gives triacetylemodic acid, identified by its conversion into free emodic acid, its methyl ester and triacetyl methyl ester. On reduction with hydriodic acid and red phosphorus in glacial acetic acid solution by the method used by Oesterle [1911] for the reduction of aloe-emodin (4:5-dihydroxy-2-(hydroxymethyl)-anthraquinone) to chrysophanic acid (4:5-dihydroxy-2-methylanthraquinone), ω-hydroxyemodin (III) is converted into *Frangula*-emodin (II). These facts can only be explained logically by assuming that ω-hydroxyemodin only differs from emodic acid (I) and *Frangula*-emodin (II) in having a CH_2OH group in the same position as the COOH group of emodic acid and as the CH_3 group of *Frangula*-emodin, and that it has, in fact, the molecular constitution 4:5:7-trihydroxy-2-(hydroxymethyl)-anthraquinone (III).



I



II



III

It is of some interest to note that *Frangula*-emodin (4:5:7-trihydroxy-2-methylanthraquinone), ω-hydroxyemodin (4:5:7-trihydroxy-2-(hydroxymethyl)-anthraquinone) and emodic acid (4:5:7-trihydroxyanthraquinone-2-carboxylic acid), all of which have now been obtained from natural sources, bear exactly the same relationship to each other as do the naturally occurring substances,

chrysophanic acid (4:5-dihydroxy-2-methylanthraquinone), aloe-emodin (4:5-dihydroxy-2-(hydroxymethyl)-anthraquinone) and rhein (4:5-dihydroxyanthraquinone-2-carboxylic acid).

While this investigation was in progress our attention was called to a recent communication [Posternak, 1939] on the pigments of *P. rubrum* Grasberger-Stoll and of *P. citreo-roseum* Dierckx. This paper gives a short preliminary account of the isolation of a pigment from *P. citreo-roseum* which has the formula $C_{15}H_{10}O_6$, melts at 273°, gives a tetraacetate, M.P. 187°, and from its general behaviour was believed by Posternak to be a polyhydroxyquinone. We were struck by the resemblance between this pigment and our ω-hydroxyemodin, and through the courtesy of Dr Posternak, whom we wish to thank, have exchanged specimens of the two pigments and of their acetates. Direct comparison of these specimens by both Dr Posternak and ourselves leaves no doubt that ω-hydroxyemodin from *P. cyclopium* and Dr Posternak's unnamed pigment from *P. citreo-roseum* are one and the same substance.

Experimental

Culture

The culture used throughout this work was isolated here in October 1938 by Dr T. Richards from mouldy gelatin. It bears Dr Richard's catalogue No. B 521 and the L.S.H.T.M. Cat. No. P 214.

We are indebted to our colleague, Mr G. Smith, for the following description of the organism. On Czapek agar (with 3% sucrose) colonies grow rapidly, spreading widely, blue-green turning duller with age, with fairly broad white margin, surface almost velvety but showing distinct granulation especially near the edges; drops large, colourless; reverse becoming faint brown from the centre outwards, becoming indistinctly zonate with age.

On wort agar colonies are slightly bluer but usually otherwise similar. On thin layers of medium, and with cultures exposed to daylight, the reverse colour is yellowish and occasionally bright yellow.

Under the microscope colonies show distinct fasciculation but no definite coremia. Conidiophores are faintly roughened, 3·2–4μ in diameter; penicilli compact, 3 times verticillate, normally with 2–3 unequal rami; conidia globose, smooth, 3μ in diameter.

The morphological findings correspond very closely with the diagnosis of *P. cyclopium* Westling given by Thom [1930], and, in culture on Czapek agar, P 214 is morphologically indistinguishable from *P. cyclopium*, L.S.H.T.M. Cat. No. P 123, obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland in 1931 and originally contributed to Baarn by Dr Charles Thom. A culture of P 214 was sent to Dr Thom who reported "It has been examined several times and morphologically your identification (as *P. cyclopium* Westling) is satisfactory."

Cultural conditions

The culture medium used throughout was a Raulin-Thom solution of the following composition: glucose, 75 g.; tartaric acid, 4·0 g.; ammonium tartrate, 4·0 g.; $(NH_4)_2HPO_4$, 0·6 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $(NH_4)_2SO_4$, 0·25 g.; $ZnSO_4$, $7H_2O$, 0·07 g.; $FeSO_4$, $7H_2O$, 0·07 g.; distilled water to 1500 ml. This medium was distributed in 350 ml. amounts in batches of 100 one-litre conical flasks plugged with cotton wool, sterilized and sown with a spore suspension of *P. cyclopium*, P 214, grown for 1–2 weeks on beer-wort agar.

It was found that, when the flasks were incubated at 24° in the dark, pigment formation was very erratic, the growth obtained being often white with little evidence of colour in the reverse. Exposure to light appears to be essential for maximum pigment production and the optimum conditions proved to be to incubate the flasks in the open laboratory at an average temperature of 20–21°. Under these conditions about 5 times as much colouring matter was isolated as was obtained from duplicate flasks incubated at 24° in the dark.

Under the optimum conditions described the mould grew quickly and after 5 days the whole surface of the culture solution was covered with a thickly sporing, bright blue-green felt having an intensely yellow or orange reverse. The metabolism solution also gradually became yellow in colour but the bulk of the colouring matter remained in the mycelium. On shaking the mycelium with dilute aqueous sodium carbonate the yellow-orange reverse changes to a brilliant crimson colour and much of the pigment dissolves out giving a crimson solution.

The production of the orange-yellow colouring matter, at any rate in appreciable amounts, appears to be peculiar to the strain of *P. cyclopium*, Cat. No. P 214. This strain, together with three other authentic strains of *P. cyclopium*, were grown side by side on Raulin-Thom solution at room temperature in daylight. The other strains used were:

(*a*) *P. cyclopium*, L.S.H.T.M. Cat. No. P 123. Obtained from Baarn in 1931.

(*b*) *P. cyclopium*, L.S.H.T.M. Cat. No. B.B 143. Isolated by Mr G. Smith from rotting tulip bulbs in 1935.

(*c*) *P. cyclopium*, L.S.H.T.M. Cat. No. H.F. Isolated by Mr G. Smith from Kentish hops in 1936.

After 14 days' growth the mycelium and metabolism solution, in the case of P 214, had become bright yellow in colour. None of the other strains showed this bright yellow reverse but instead varied in colour from pale buff to purplish-brown with the metabolism solutions pale dirty yellow to brown. On treatment with dilute aqueous sodium carbonate there was in each case only a slight reddening which was not comparable with the intense crimson given by P 214. Extraction of the mycelia with dilute HCl and ether gave faintly yellow ethereal solutions which turned brown on shaking with aqueous sodium carbonate, whereas P 214 gave an intense crimson colour in the carbonate solution.

Isolation of the crude colouring matters

At the end of the incubation period the mycelium from each batch of 100 flasks was separated from the metabolism solution, which was discarded, by straining through butter muslin. The total mycelium was divided into two approximately equal parts. Each part was placed in a 10 l. bottle together with 5 l. of ether and 2·5 l. of 2*N* HCl. The contents of each bottle were vigorously stirred for 1 working day with a mechanical stirrer and were then set aside overnight. The ether-acid layer was drained from the mycelium, which was washed with about 1 l. of fresh ether. The combined ether extract and washings were separated from the acid layer, filtered, washed with water and dried over anhydrous $MgSO_4$. The golden-yellow ethereal solution was evaporated to 100–150 ml. and set aside for 2–3 hr. The golden-brown solid which separated was filtered and washed with a little ether, giving Fraction I consisting of a dry, golden-brown powder which did not melt completely below 310°. The ether mother liquors were evaporated to dryness giving a somewhat sticky residue which was extracted with boiling light petroleum (20–30 ml., B.P. 50–60°), filtered and dried giving Fraction II, which consisted of a dry brown powder melting with much decomposition and blackening between 290° and 310°.

The method of extraction described gives complete extraction of all colouring matters and the extracted mycelium no longer gives a colour with dilute sodium carbonate. It is much superior to other methods tried in preliminary experiments, e.g. extraction of the dried mycelium with ether in a Soxhlet apparatus or extraction of the undried mycelium with dilute sodium carbonate and acidification of the extract.

The experimental details of five representative batches, all of which were grown in the open laboratory, follow. In all cases all flasks were covered with a thick, crinkled, green, heavily sporing mycelium with a striking golden reverse. Average temperature, 20–21°. (Limits during night and day, 17–23°.)

Incubation period in days	16	19	18	17	18
Residual sugar by polarimeter, %	0·71	0·52	0·42	0·44	0·41
Yields of Fraction I, g.	5·4	5·1	5·5	4·6	5·3
Yields of Fraction II, g.	1·2	1·1	0·5	1·0	0·6

Fractionation of the crude colouring matters

Fraction I of the crude colouring matters consists essentially of a mixture, in about equal amounts, of emodic acid (4:5:7-trihydroxyanthraquinone-2-carboxylic acid) and the hitherto undescribed *ω-hydroxyemodin* (*4:5:7-trihydroxy-2-(hydroxymethyl)-anthraquinone*). Fraction II is a much cruder product containing a bigger proportion of emodic acid. In the earlier work these two substances were separated from Fraction I by a very laborious series of fractionations, first from ethanol and then from glacial acetic acid. A much simpler and more satisfactory method of separation depends on the fractionation of their acetates and regeneration of the parent substances by alkaline hydrolysis of the purified acetates.

A typical example of this method was carried out as follows: The mixture of crude colouring matters (2·0 g.) was suspended in acetic anhydride (20 ml.) and conc. H_2SO_4 (0·4 ml.) was added drop by drop. The pigment quickly dissolved to give a yellowish-brown solution which was warmed on the water bath for a few moments and was then boiled under a reflux condenser for 2 min. The cooled mixture was poured into ice-water. The brown oil which separated quickly crystallized and was filtered, washed and dried (2·8 g.). This material was dissolved in boiling ethanol (180 ml.), norite (0·5 g.) was added and the mixture was filtered and cooled. A woolly mass of pale yellow needles of almost pure *tetraacetyl-ω-hydroxyemodin* very quickly separated (1·06 g.) which, on recrystallization from ethanol, had a constant M.P. of 190–191°. The ethanol filtrate was evaporated to 40 ml. and, on standing, slowly deposited brownish-yellow irregular plates (1·12 g.) which, on recrystallization from glacial acetic acid (with norite) gave bright canary-yellow prisms or plates of triacetylemodic acid, M.P. 218–219°. A further small amount of crude triacetylemodic acid was obtained from the evaporated ethanol mother liquors.

The total yields obtained from five batches of 100 flasks per batch were: (*a*) Fraction I of crude colouring matters, 25·9 g., giving 34·7 g. of crude mixed acetates, from which were isolated 11·5 g. of tetraacetyl-ω-hydroxyemodin and 13·8 g. of triacetylemodic acid. (*b*) Fraction II of crude colouring matters, 4·4 g., giving 5·5 g. of crude mixed acetates from which were isolated 1·0 g. of tetraacetyl-ω-hydroxyemodin and 2·1 g. of triacetylemodic acid.

(A) *ω-Hydroxyemodin* (*4:5:7-trihydroxy-2-(hydroxymethyl)-anthraquinone*)

(1) *Preparation of ω-hydroxyemodin from its acetate.* Tetraacetyl-ω-hydroxyemodin (3 g., M.P. 190°) was heated with aqueous 2 *N* NaOH (150 ml.) on a boiling water bath for 2 hr. in an atmosphere of nitrogen. The acetate quickly dissolved,

giving finally an intensely reddish-purple solution which, at the end of the hydrolysis period, was acidified with 2*N* HCl (225 ml.). The acidified mixture was heated for 30 min. to coagulate the gelatinous orange-yellow precipitate which was then separated by filtration, washed free from chlorides and dried. The dark brown solid (1·97 g.) was crystallized from methanol (300–400 ml.) with norite giving beautifully crystalline, dull orange, slender rods or needles (1·5 g.), M.P. 287–288°. The M.P. was raised to 288° (constant) by further recrystallization from methanol. (Found: on material dried to constant weight in a high vacuum at 120°, loss in wt. 7·0 %. C, 62·78, 62·61; H, 3·57, 3·72; N, nil; CH_3O, nil. $C_{15}H_{10}O_6$ requires C, 62·91; H, 3·52 %.)

ω-Hydroxyemodin may also be prepared from its acetate by boiling with methanol containing 3 % by volume of concentrated H_2SO_4.

ω-Hydroxyemodin is insoluble in cold 2 % aqueous $NaHCO_3$; readily soluble in cold *N* Na_2CO_3 and in *N* NaOH, the solution being in each case red in the bulk with a bluish shade in thin layers; with cold conc. H_2SO_4 it gives a reddish-orange colour in bulk with an eosin-like shade in thin layers. Emodin (4:5:7-trihydroxy-2-methylanthraquinone) behaves similarly with the above reagents and gives colours with them which are almost indistinguishable from those given by ω-hydroxyemodin.

(2) *Tetraacetyl-ω-hydroxyemodin* (*4:5:7:2′-tetraacetoxy-2-methylanthraquinone*). This acetate, whether prepared by fractionation of the crude mixed acetates as described above or by direct acetylation of ω-hydroxyemodin prepared by fractionation of the mixed colouring matters themselves, forms very characteristic pale yellow, soft, woolly needles, M.P. 190–191°, which are quite insoluble in either cold 2*N* Na_2CO_3 or in 2*N* NaOH. (Found: C, 60·81, 60·83; H, 3·98, 4·03; CH_3CO, 35·2, 35·0 %. $C_{23}H_{18}O_{10}$, i.e. $C_{15}H_6O_2$ $(O.CO.CH_3)_4$ requires C, 60·77; H, 4·00; $4CH_3CO$, 37·9 %.)

(3) *ω-Hydroxyemodin monomethyl ether* (*4:5-dihydroxy-7-methoxy-2-(hydroxymethyl)-anthraquinone*). A mixture of ω-hydroxyemodin (0·54 g.), methyl iodide (0·9 g.) and sodium methoxide (0·14 g. of sodium dissolved in 10 ml. of methanol) was heated in a sealed tube for 4 hr. in a boiling water bath. The initially intensely purple solution changed to a brownish orange in colour and deposited, on cooling, 0·38 g. of crystalline material which was crystallized from ethanol in pale dull orange plates which are paler and duller in colour than the parent substance. M.P. 229–231°. (Found: C, 63·93; H, 4·09; CH_3O, 10·0 %. $C_{16}H_{12}O_6$ requires C, 63·98; H, 4·03; $1CH_3O$, 10·3 %.) The substance is quite insoluble in cold 2 % aqueous Na_2CO_3 but dissolves in *N* NaOH and conc. H_2SO_4 giving solutions which are indistinguishable in colour from those given by the parent substance. Its constitution as the 7-methyl ether of ω-hydroxyemodin follows from its insolubility in dilute Na_2CO_3 and from its method of preparation which is that used for the methylation of β-hydroxy groups in polyhydroxyanthraquinones, α-hydroxy groups not being methylated under these conditions (cf. the conversion of *Frangula*-emodin into its 7-methyl ether, physcion [Jowett & Potter, 1903; Tutin & Clewer, 1910]).

(4) *Oxidation of ω-hydroxyemodin to emodic acid* (*4:5:7-trihydroxyanthraquinone-2-carboxylic acid*). Tetraacetyl-ω-hydroxyemodin was oxidized to triacetylemodic acid by the method described by Fischer & Gross [1911] for the oxidation of triacetylemodin to triacetylemodic acid. Tetraacetyl-ω-hydroxyemodin (2 g.) was dissolved at 50–60° in a mixture of glacial acetic acid (40 ml.) and acetic anhydride (40 ml.). The temperature was maintained at 50–60° while a mixture of chromic anhydride (4 g.) dissolved in water (3·2 ml.) and glacial acetic acid (40 ml.) was gradually added over 30 min. with constant

shaking. The bath was then raised to 65–70° and kept at this temperature for 3 hr. when a pure deep green colour had developed. The solution was now poured into hot water (1200 ml.) and left in the cold room overnight. The yellow crystalline product which had separated was filtered, washed and dried. Wt. 1·44 g., M.P. 218–219°. The product is almost pure triacetylemodic acid since a specimen of this substance made by oxidizing triacetylemodin by the same method (see below) melted at the same temperature, as did a mixture of the two acetates. Contrary to what might be expected on theoretical grounds tetra-acetyl-ω-hydroxyemodin appears to be rather more difficult to oxidize with chromic acid than is triacetylemodin.

The oxidation acetate (1·44 g.), without further purification, was hydrolysed by heating with 2*N* NaOH (75 ml.) on a boiling water bath for 2 hr. in an atmosphere of nitrogen. The deep reddish-purple solution was then acidified with 2*N* HCl (100 ml.), heated for 30 min. more to coagulate the orange gelatinous precipitate, filtered, washed free from chlorides and dried. Wt. 0·91 g. This dark brown amorphous solid was crystallized twice from glacial acetic acid (about 300 ml.) from which it separates, with very little loss, in shining orange needles. The product, emodic acid, (4:5:7-trihydroxyanthraquinone-2-carboxylic acid) behaves in a characteristic manner on heating. No change is obvious up to 340°, but at about 350° the substance begins to smoke and finally melts with decomposition at about 363–365°. The same behaviour was seen on heating synthetic emodic acid prepared from emodin, and mould emodic acid (see below) and with mixtures of either of these specimens with the oxidation product. (Found, on material dried in a boiling xylene bath: Loss in wt., 15·9 %. Theoretical for loss of 1 mol. of acetic acid, 16·7 %. C, 59·95, 59·86; H, 2·64, 2·77 %. Calculated for $C_{15}H_8O_7$, C, 59·99; H, 2·69 %.)

In order to avoid any dubiety as to the identity of the oxidation product with synthetic emodic acid, arising out of their high M.P., the *methyl ester* and *triacetyl-methyl* ester were prepared.

(5) *Methyl ester of the oxidation product* (*methyl emodate*). For method of preparation see p. 167. 0·7 g. of the oxidation product gave 0·57 g. of crude methyl emodate, M.P. 266–267°, which, on recrystallization from methanol, gave reddish-orange needles (0·53 g.), M.P. 268–269°, alone or in admixture with the methyl ester of synthetic emodic acid. (Found: C, 60·65; H, 3·38; CH_3O, 10·15, 9·7 %. $C_{16}H_{10}O_7$ requires C, 61·13; H, 3·21; $1CH_3O$, 9·9 %.)

(6) *Triacetate of the methyl ester of the oxidation product* (*methyl triacetyl emodate*). For method of preparation see p. 167. 0·3 g. of the above methyl ester gave 0·3 g. of recrystallized triacetate. Fine, pale yellow needles, *ex* glacial acetic acid, M.P. 188°. A mixture with the triacetyl methyl ester of synthetic emodic acid, M.P. 188–189°, melted at 188–189°. (Found: C, 60·24, 59·98; H, 3·82, 3·98; CH_3O, 7·4, 7·2; CH_3CO, 29·6, 29·3 %. $C_{22}H_{16}O_{10}$ requires C, 59·98; H, 3·66; $1CH_3O$, 7·05; $3CH_3CO$, 29·3 %.)

(7) *Reduction of ω-hydroxyemodin to* Frangula-*emodin* (*4:5:7-trihydroxy-2-methylanthraquinone*). A mixture of ω-hydroxyemodin (1 g.), glacial acetic acid (20 ml.), hydriodic acid (4 ml. sp. gr. 1·7) and red phosphorus (1 g.) was boiled under reflux for 4 hr. The reaction mixture was cooled, poured into water (200 ml.) and the pale brown colour of the solution was discharged by the addition of a slight excess of sodium bisulphite. After standing overnight the purple-brown precipitate was filtered, washed and dried. It was extracted with boiling acetic acid (about 200 ml.) and from the extract there was obtained a total of 0·72 g. of sand-coloured material which appeared under the microscope as almost colourless flat rectangular plates which, on heating, showed no change up to

250°, began to darken at 250–255°, and decomposed completely with intense blackening at 255–258°. (Jacobson & Adams [1924] give a decomposition point of 250–258° for emodin anthranol, 4:5:7-trihydroxy-2-methylanthranol, and Tutin & Clewer [1912] about 255°.)

The anthranol (0·71 g.) was dissolved in boiling glacial acetic acid (200 ml.), the solution cooled to 60°, and to it was quickly added a solution of chromium trioxide (0·75 g.) in water (10 ml.) and glacial acetic acid (10 ml.). The dark-brown reaction mixture was maintained at 60° for 30 min., and was then evaporated *in vacuo* to 25 ml. On standing, emodin (0·46 g.) separated in cinnamon-coloured rods, M.P. 255–256°. On recrystallization from toluene (norite), pure emodin was obtained in dull orange needles, M.P. 256–257°, not depressed on admixture with an authentic specimen of *Frangula*-emodin. (Found on material dried in a boiling xylene bath: C, 66·69, 66·74; H, 3·84, 3·61 %. Calc. for $C_{15}H_{10}O_5$, C, 66·64; H, 3·73 %.)

The identity of the reduction product was confirmed by conversion into the triacetate, yellow needles, *ex* ethanol, M.P. 198°, alone or in admixture with triacetylemodin.

(B) *Mould emodic acid (4:5:7-trihydroxyanthraquinone-2-carboxylic acid)*

(1) *Preparation of mould emodic acid from its acetate.* The acetate (3·0 g.) having the higher M.P. 218–219°, and obtained as the more soluble fraction of the mixture of acetates from the acetylation of the crude colouring matters, was heated with aqueous 2*N* NaOH (150 ml.) on a boiling water bath for 2 hr. in an atmosphere of nitrogen. The intensely reddish-purple hydrolysis solution was acidified with 2*N* HCl (210 ml.) and heated for 30 min. more to coagulate the orange-yellow gelatinous precipitate which was filtered, washed free from chlorides and dried (1·98 g.). This material was crystallized twice from glacial acetic acid (400–450 ml.), with norite, giving a mass of glistening orange needles, which show no change on heating up to 340°, smoke at about 350° and melt with decomposition at 362–363°. The M.P. was not depressed on admixture either with synthetic emodic acid from emodin or with the hydroxyanthraquinone, M.P. 360°, prepared by direct fractional crystallization from glacial acetic acid of the crude mixture of colouring matters. (Found: (*a*) on material dried to constant wt. in a high vacuum at 110°, loss in wt. 12·7 %. C, 56·60, 56·48; H, 3·20, 3·19 %. CH_3O, nil. $C_{15}H_8O_7$, H_2O ($C_{15}H_{10}O_8$) requires C, 56·59; H, 3·17 %. (*b*) On material dried in a boiling nitrobenzene bath at 210°, loss in wt. 15·9 %. C, 59·78, 59·67; H, 2·85, 2·84 %. Calc. for $C_{15}H_8O_7$, C, 59·99; H, 2·69 %.)

Hydrolysis of the acetate (0·1 g.), M.P. 218–219°, with methanol (5 ml.) containing 3 % by volume of conc. H_2SO_4 for 2 hr. led to the formation, not of emodic acid, but of its methyl ester (0·06 g.), which crystallized from chloroform in glistening orange rectangular leaflets, M.P. 268°. (Found: C, 60·77, 60·77; H, 3·24, 3·17; CH_3O, 9·75 %. $C_{16}H_{10}O_7$ requires C, 61·13; H, 3·21; $1CH_3O$, 9·9 %.)

Mould emodic acid is readily soluble in cold 2 % aqueous $NaHCO_3$ giving a reddish-orange solution. It readily dissolves in *N* Na_2CO_3 and in *N* NaOH giving in each case a solution which is cherry-red in bulk with a blue shade in thin layers. With cold conc. H_2SO_4 it gives a reddish-orange colour when seen in bulk, with an eosin-like shade in thin layers, this colour being indistinguishable from that given by ω-hydroxyemodin with conc. H_2SO_4. Synthetic emodic acid from emodin and emodic acid from the oxidation of ω-hydroxyemodin behave similarly with the above reagents and give colours with them which are indistinguishable from each other.

(2) *The triacetate of mould emodic acid.* This acetate, whether prepared by the direct acetylation of mould emodic acid with acetic anhydride and conc. H_2SO_4 or by fractionation, as previously described, of the mixed acetates from the crude mould colouring matters, forms bright canary-yellow blunt-ended prisms from glacial acetic acid, which give a deep red solution in cold dilute Na_2CO_3. Its M.P., which varies somewhat with the rate of heating, is 218–219° and is not depressed on admixture with triacetylemodic acid obtained either by the oxidation of triacetyl emodin (see below) or by the oxidation of tetraacetyl-ω-hydroxyemodin (see p. 165). (Found: on material dried to constant wt. at 110° in a high vacuum, loss in wt. 12·80%; loss of 1 mol. acetic acid requires 12·35%. C, 58·75; H, 3·56; CH_3CO, 28·7%. Calc. for $C_{21}H_{14}O_{10}$, C, 59·14; H, 3·31. $3CH_3CO$, 30·3%.)

(3) *The methyl ester of mould emodic acid.* For method of preparation see below. 0·72 g. of mould emodic acid gave 0·49 g. of the methyl ester which on recrystallizing from methanol gave shining orange-red needles (0·39 g.), M.P. 270°. A mixture with synthetic methyl emodate (M.P. 268–269°) melted at 268–269°. (Found: CH_3O, 10·45%. $C_{16}H_{10}O_7$ requires for $1CH_3O$, 9·9%.)

(4) *The triacetate of the methyl ester of mould emodic acid.* For method of preparation see below. 0·2 g. of the above methyl ester gave 0·26 g. of acetate, which on crystallization from glacial acetic acid gave fine, pale yellow needles, M.P. 187–188°, alone or in admixture with the triacetyl methyl ester of synthetic emodic acid. (Found: C, 59·51; H, 3·57; CH_3O, 7·35%. $C_{22}H_{16}O_{10}$ requires C, 59·98; H, 3·66; $1CH_3O$, 7·05%.)

(C) *Synthetic emodic acid (4:5:7-trihydroxyanthraquinone-2-carboxylic acid) and its derivatives*

Emodin (4:5:7-trihydroxy-2-methylanthraquinone, M.P. 255°, supplied by Schering-Kahlbaum A.G. Berlin) (5 g.) was acetylated by heating at 60–70° for 30 min. with acetic anhydride (50 ml.) and conc. H_2SO_4 (1 ml. added drop by drop). The crude triacetylemodin obtained by pouring the acetylation mixture into ice-water was dried (7·0 g., M.P. 193–195°) and oxidized, according to the method of Fischer & Gross [1911] with chromic acid in a mixture of acetic anhydride and glacial acetic acid. Yield of crude triacetylemodic acid, 5·97 g., M.P. 218–219°. This almost pure product was hydrolysed with aqueous 2*N* NaOH to emodic acid which was crystallized from glacial acetic acid. Yield of recrystallized emodic acid, 4·11 g. Orange needles, M.P. 364–365° (decomp.) after smoking at about 350°. The details, modified for quantities, of the oxidation and hydrolysis are the same as those described for the oxidation of tetraacetyl-ω-hydroxyemodin and the hydrolysis of the resulting triacetylemodic acid (see p. 164).

Methyl emodate. A mixture of synthetic emodic acid (1 g.), methanol (100 ml.) and conc. H_2SO_4 (3 ml.) was refluxed for 3·5 hr. The emodic acid slowly dissolved (1·5 hr.) and, on standing, the esterification mixture deposited crystals of almost pure ester (0·84 g.), which were recrystallized from methanol. Glistening reddish-orange needles, M.P. 268°. (Found: C, 60·87, 61·00; H, 3·22, 3·40; CH_3O, 9·4, 9·3%. $C_{16}H_{10}O_7$ requires C, 61·13; H, 3·21; $1CH_3O$, 9·9%.)

The ester is insoluble in cold 2% aqueous $NaHCO_3$ but readily dissolves in cold *N* Na_2CO_3 and in cold conc. H_2SO_4 giving colours with these reagents which are indistinguishable from those given by emodic acid itself.

Triacetylemodic acid methyl ester. Methyl emodate (0·8 g.) was suspended in acetic anhydride (10 ml.) and conc. H_2SO_4 (5 drops) was added. On warming the mixture gently on the water bath the ester dissolved, giving a clear pale yellow

solution from which, on cooling, the acetate quickly separated in pale yellow needles (0·81 g.) which were filtered, washed with glacial acetic acid and recrystallized from this solvent. A further 0·19 g. of the acetate was obtained on pouring the acetic anhydride mother liquors into water. This acetate, which is very suitable for the identification of emodic acid, crystallizes from glacial acetic acid in fine, pale yellow needles which melt sharply at 188–189°, the melt resetting on cooling. (Found: C, 60·08, 60·30; H, 3·57, 3·78; CH_3O, 6·9, 7·0; CH_3CO, 28·3, 28·0 %. $C_{22}H_{16}O_{10}$ requires C, 59·98; H, 3·66; $1CH_3O$, 7·05; $3CH_3CO$, 29·3 %.)

Summary

Emodic acid (4:5:7-trihydroxyanthraquinone-2-carboxylic acid) and the hitherto undescribed *ω-hydroxyemodin* (*4:5:7-trihydroxy-2-(hydroxymethyl)-anthraquinone*) have been isolated from the mycelium of a strain of *Penicillium cyclopium* Westling grown in daylight at 20–21° on Raulin-Thom solution.

We wish to thank the Chemistry Research Board of the Department of Scientific and Industrial Research for a research grant which has made this work possible.

REFERENCES

Asahina & Fuzikawa (1935). *Ber. dtsch. chem. Ges.* **68**, 1558.
Ashley, Raistrick & Richards (1939). *Biochem. J.* **33**, 1291.
Charles, Raistrick, Robinson & Todd (1933). *Biochem. J.* **27**, 499.
Eder & Hauser (1925). *Helv. chim. Acta*, **8**, 126.
—— & Widmer (1923). *Helv. chim. Acta*, **6**, 966.
Fischer & Gross (1911). *J. prakt. Chem.* (II), **84**, 369.
Jacobson & Adams (1924). *J. Amer. chem. Soc.* **46**, 1312.
Jowett & Potter (1903). *J. chem. Soc.* **77**, 1330.
Oesterle (1911). *Arch. Pharm., Berl.*, **249**, 445.
Posternak (1939). *C.R. Soc. Phys. Hist. nat. Genève*, **56**, 28.
Raistrick, Robinson & Todd (1933). *Biochem. J.* **27**, 1170.
—— —— —— (1934). *Biochem. J.* **28**, 559.
—— —— —— (1937). *J. chem. Soc.* p. 80.
Thom (1930). The Penicillia. London: Baillière, Tindall and Cox.
Tutin & Clewer (1910). *J. chem. Soc.* **97**, 3.
—— —— (1912). *J. chem. Soc.* **101**, 299.

27. STUDIES IN IMMUNOCHEMISTRY

4. THE FRACTIONATION AND NATURE OF ANTIGENIC MATERIAL ISOLATED FROM *BACT. DYSENTERIAE* (SHIGA)[1]

By W. T. J. MORGAN and S. M. PARTRIDGE

From the Biochemical Department, Lister Institute of Preventive Medicine, London

(*Received 22 December 1939*)

The work of Boivin *et al.* [1933; 1934; 1935; 1937], Raistrick & Topley [1934], Topley *et al.* [1937], Mesrobeanu [1936], Morgan [1937], Henderson & Morgan [1938] and Miles & Pirie [1939, 1, 2, 3] has shown that the antigens of certain Gram-negative micro-organisms can be isolated in a relatively pure condition and apparently unchanged in their specific immunological properties. A method for the isolation of the antigenic complex from two such organisms—*Bact. dysenteriae* (Shiga) and *Bact. typhosum*—by extraction of the dry bacteria with anhydrous diethyleneglycol has already been described by Morgan [1937] and Henderson & Morgan [1938]. In the present communication the results of further work based on material isolated by the use of this method are given and evidence as to the chemical and physical nature of the dominant somatic antigen of the "smooth" strain of *Bact. dysenteriae* (Shiga) is discussed.

Before a more detailed investigation into the chemical nature of the antigenic material could be profitably undertaken it was considered advisable to demonstrate as conclusively as possible the homogeneous nature of the antigenic preparation selected for study and with this aim in view a number of experimental procedures were elaborated and applied. It would appear, however, to be an open question whether our present-day knowledge of the chemistry and physics of large molecular complexes will enable a decision on this point to be reached. In consequence it will be appreciated that in the absence of any recognized technique whereby naturally occurring colloidal complexes of similar nature may be separated from one another, some of the conclusions reached concerning the nature of the specific antigen may need subsequent modification. In the meantime, as further work on this subject is unavoidably postponed, the results of the investigation so far as completed are set out in this paper. A preliminary account has already appeared; Morgan & Partridge [1939, 2].

Extraction and purification of the antigenic complex

The method used for the extraction of the antigenic complex has been described [Morgan, 1937]; additional details are now given. The preparation of bacteria of the "smooth" strain of *Bact. dysenteriae* (Shiga) was extracted 4 times with about 10 times its weight of anhydrous diethyleneglycol (b.p. 244–246°). Each extraction period lasted 4 days. The glycol suspension of the bacteria was mixed with 0·1 vol. of methyl alcohol, in order to reduce the density and

[1] For Part III see Morgan [1938, 1].

viscosity of the fluid medium and was centrifuged free from organisms in a Sharples supercentrifuge. A clear supernatant fluid which would readily pass through a British Berkefeld candle could usually be obtained after centrifuging for 1 hr. at about 35,000 r.p.m. After filtration the diethyleneglycol solution of the antigen was dialysed at 0° to eliminate the organic solvent and the antigenic material was obtained as a colloidal aqueous solution. The material was concentrated *in vacuo* at 10–15° until the solid content was between 1 and 2% whereupon the solution was centrifuged to remove any insoluble matter, cooled to 0° and treated with oxalic acid until no further insoluble material separated after standing at 0° for 24 hr. The deposit was removed by centrifuging and the excess oxalic acid was eliminated by dialysis against ice-cold distilled water. The antigen solution was treated at 0° with an equal volume of acetone which had been previously cooled to −10°; only a small quantity of material usually separated. The centrifuged solution was then treated with a volume of cold acetone equal to that of the aqueous antigen solution which immediately caused the precipitation of the main part of the antigenic material. The supernatant solution (66% acetone) was concentrated *in vacuo* and subsequently worked up for further antigen recovery when several batches of material became available. In order to eliminate the agar-like products that are present in the original antigen preparation at least two further acetone fractionations, between the levels 50 and 60%, were carried out on the redissolved 50–66% acetone precipitate. The material was then collected by centrifuging, resuspended in distilled water and dialysed free from acetone; it was ultimately obtained in the solid form by freezing the aqueous solution at −20° and allowing the frozen material to evaporate at room temperature *in vacuo* over H_2SO_4. Antigenic material prepared in this manner was used in all the experimental procedures that will now be described.

Before passing on, however, it is of interest to record the results obtained in diethyleneglycol extraction experiments in which several hundred g. of dry bacteria have been used. Earlier experiments [Morgan, 1937] have already shown that the material prepared as described above contains almost the whole of the specific antigenic complex present in the organism. It will be seen from the results summarized in Table 1 that the yield of antigenic material extracted

Table 1. *Antigenic material extractable with diethyleneglycol from the "smooth" form of* Bact. dysenteriae (*Shiga*)

Exp. no.	19	20	21	26
Total weight of dry bacteria (g.)	50	50	64	60
Extraction no. 1	1·29	1·59	2·00	2·10
,, 2	0·69	0·53	0·67	1·90
,, 3	0·22	0·30	0·65	0·58
,, 4	0·14	0·20	—	0·15
Total material extracted (g.)	2·34	2·62	3·32	4·73
,, ,, (% on dry bacteria)	4·7	5·2	5·2	7·8

by diethyleneglycol and purified by subsequent fractionation between the limits 50 and 60% acetone falls off rapidly with each successive treatment. Three successive treatments with diethyleneglycol, each of 4 days' duration, removed approximately 60, 25 and 12% respectively of the total antigen extractable under these conditions. The results also show that 5–7% by weight of the dried bacteria is recovered by this procedure and it would appear that this figure represents approximately the amount of antigen present in the dry organism.

Further extraction with diethyleneglycol, especially after prolonged contact lasting up to several weeks, yields some more material but the properties of the extracted substance show it to be quite distinct from the specific antigen of the "smooth" strain. This additional material, which evidently resides deep in the body of the organism, can only be reached after the surface antigen has been removed and is most probably, in part at least, an antigen characteristic of the "rough" strain. The yield of this material, even after prolonged extraction, amounts to little more than 1 % of the dry weight of the organisms extracted, but its presence in a diethyleneglycol extract suggested that a preparation of bacteria of the corresponding "rough" strain (K 624) should be subjected to a diethyleneglycol extraction process identical with that employed for the preparation of the specific "smooth" antigen. Accordingly, for control purposes, an extraction experiment was carried out with 45 g. of dry "rough" *Bact. dysenteriae* (Shiga) which is known to lack the specific antigen of the "smooth" strain. The material extractable from the "rough" strain is composed of "rough" antigen [Meyer, 1930] and certain non-specific material which is derived from the body substance of the organism. These substances are presumably also components of the "smooth" organism. Table 2 shows clearly that the amount

Table 2. *Material extractable with diethyleneglycol from "smooth" and "rough" variants of* Bact. dysenteriae (*Shiga*). (*Strain K 624*)

	"Smooth"	"Rough"
	g.	g.
Weight of organisms	77	45
1st extract	2·7	0·09
2nd extract	1·1	Trace
3rd extract	0·25	Trace
4th extract	0·03	—
Total material extracted	4·08	0·09
Total material extracted (% of dry organisms)	5·3	0·2

of material extractable from the "rough" strain, after the usual fractionation of the product from aqueous solution by acetone, is quite small, being of the order of 0·2 % of the weight of the organisms extracted. If it is assumed that a similar amount of material, other than "smooth" antigen, is likewise dissolved from the organisms of the "smooth" strain and is subsequently present in the antigen preparation, then the extent of contamination of the "smooth" antigen with "rough" antigen and with non-specific somatic material amounts at most to 3 or 4 %.

Fractionation with acetone. A preparation of antigen from the "smooth" strain obtained by diethyleneglycol extraction of the dry bacteria and fractionated twice with acetone between the levels 50 and 66 % by volume was repeatedly fractionated from 1 % aqueous solution with cold acetone in order to ascertain whether such treatment enabled the material to be separated into components that were chemically or immunologically distinct.

The antigenic material was suspended in distilled water to yield a 1 % colloidal solution. The opalescent solution was cooled to 0° and acetone at −10° was slowly added with constant shaking. The addition of an equal volume of acetone produced no visible precipitate but centrifuging caused about 5 or 10 % of substance to separate. When the acetone concentration reached 60 % by volume a large precipitate of the antigenic material separated and was collected by centrifuging. The supernatant fluid was made up to 66 % with acetone, a further fraction was removed and the acetone solution was concentrated under

reduced pressure at 20°, dialysed and dried. All fractions precipitated with acetone were resuspended in water and were dialysed at 0° to remove acetone. The fractions were then obtained in the solid form by rapid evaporation *in vacuo* of the frozen solutions. The optical rotations were measured in neutral formamide. The material precipitated by 50 % acetone, however, frequently gave an opalescent solution in formamide and for this reason the specific rotation could only be approximately determined.

The material thrown out of solution between the levels 50 and 60 % acetone was usually found to make up 70–80 % of the antigenic substance employed and showed a slight opalescence in formamide solution; $[\alpha]_{5461}+55\pm5°$ (*c*, 0·5). A small fraction, $[\alpha]_{5461}+60\pm5°$, was precipitated between 60 and 66 % acetone and not more than 5 % of the original material was found to be soluble in the 75 % acetone supernatant fluid. The main fraction was again fractionated from 1 % aqueous solution by acetone. There was usually no precipitate at 50 % acetone but a large fraction was thrown out of solution when the acetone concentration was increased from 50 to 55 %. This fraction, in 1 % aqueous solution, was treated a third time with acetone but no material separated which showed a rotation outside the limits $[\alpha]_{5461}+56\pm5°$. The antigenic material at this stage yields an almost clear, colourless solution in formamide. It was apparent that no further separation could be achieved by means of this technique and in one experiment, that involved four separate fractionation processes, 7 g. of the primary extraction product gave rise to 5·4 g. of antigenic material, $[\alpha]_{5461}+56\pm3°$, which precipitated between 50 and 55 % of acetone. Other fractions obtained during the process showed extreme rotations which varied from +45 to +62° but subsequent treatment of these again yielded a main fraction which showed $[\alpha]_{5461}+56\pm5°$ (*c*, 0·5 in formamide). A small amount of the specific polysaccharide component was found in the final acetone supernatant fluids.

Fractionation with alcohol. A similar series of fractionations was made in which absolute alcohol replaced acetone but no definite indication could be obtained that the antigenic material was composed of a simple mixture of different substances.

Fractionation with ammonium sulphate. Attempts were made to fractionate a specimen of the antigenic material by means of ammonium sulphate after the complex had been repeatedly precipitated from aqueous solution by acetone. The material (500 mg. in 50 ml. water) was almost completely thrown out of solution between the ammonium sulphate levels 21–23 % by volume. The supernatant fluid yielded only 38 mg. of antigenic material after it had been dialysed free from ammonium sulphate and evaporated to dryness. It would appear, therefore, that the antigenic material cannot be separated into several fractions that show different solubilities in ammonium sulphate solution.

Extraction with organic solvents. In view of the earlier work [Mesrobeanu & Calab, 1936; Morgan, 1937], which showed that a fatty substance could be obtained from the antigenic complex by acid hydrolysis, it seemed desirable at this juncture to prove that the fatty substance is not simply a loosely bound contaminant but is in fact a definite part of the antigenic complex. A preparation of the primary extraction product that weighed 6 g. was therefore extracted successively with (*a*) five portions each of 200 ml. of freshly distilled ether, (*b*) five portions each of 200 ml. of a 1 : 3 alcohol-ether mixture, (*c*) two 200 ml. portions of redistilled chloroform and finally a colloidal aqueous solution of the material was thoroughly extracted with freshly distilled ether. As a result of these extractions only 0·8 % of the total weight of the antigenic material

was found to be soluble. It would appear, therefore, that the fatty material which can be isolated in a yield of 9–12 % of the substance after acetic acid hydrolysis of the complex and also, as will be described later, by the action of formamide, is in fact a component of the antigen and is not merely adventitious material.

Antigenicity tests. Numerous antigenicity tests in rabbits were made with material obtained by acetone or alcohol fractionation and possessing a specific rotation in anhydrous formamide of $+56° \pm 5°$. In every preparation examined an immunizing course of three doses each of 0·01 mg. of the fraction under test gave rise to an immune-serum showing agglutination to high titre whereas precipitation, haemolytic and complement fixation tests, where these were made, indicated that the immune-serum produced was qualitatively identical with the antibacterial immune-body induced by a killed culture of a "smooth" strain of *Bact. dysenteriae* (Shiga). A more detailed account of the immunological aspects of this work will be given elsewhere.

Antigenic material that has been repeatedly precipitated from 1 % aqueous solution by acetone between the levels 50 and 60 % and which is called "the primary extraction product" appears therefore to be largely homogeneous in nature and free from non-specific substances. It cannot be stated, however, that this product is identical in its chemical and physical properties with the antigenic complex as it exists in the intact organism. Further observations on this point will be discussed later in the paper.

The properties of the primary extraction product

The substance in a concentration of 1 % gives rise to a heavy milky opalescent aqueous solution but dissolves readily in anhydrous formamide, formic acid (99–100 %) and concentrated phenol solution to give clear solutions. The material is insoluble in glacial acetic acid and dioxan. The opalescence in 6·6 *M* urea solution is considerably less than that in water; this is presumably due to disaggregation of the complex into smaller units. The antigenic material after disaggregation in strong solutions of urea or formic acid can be obtained largely in a reaggregated form by removing the urea or formic acid by dialysis and recovering the antigen in the dry condition; re-solution of the material in water again gives rise to a strongly opalescent solution. A determination of the sedimentation factors under appropriate conditions with a view to ascertaining the particle size of the primary extraction product both before and after treatment with different solvents is being undertaken in collaboration with Dr A. S. McFarlane and the relation of changes in the size of the antigen particle to the biological activity of the product is also under investigation.

A 1 % solution of the primary extraction product after heating in 1 % acetic acid at 100° for 30 min. commences to flocculate and the flocculation process is complete in about 1 hr. The same antigenic preparation, however, when heated in neutral aqueous solution under the same conditions, shows no evidence of flocculation up to a period of at least 3 hr. After this time the colloidal properties of the complex are destroyed and precipitation of the polypeptide and phospholipin components occur. The loss of antigenicity during heating appears to take place before flocculation and in one experiment antigenic material that had been heated at *p*H 7 for 1 hr. was found to induce in rabbits no demonstrable precipitins for the specific polysaccharide after the usual three intravenous doses each of 0·05 mg. had been given. It is of interest to record that the phospholipin component of the antigenic complex is not removed from the complex by extraction with ether after the material has been heated at 100° for 1 hr.,

that is, after the material has lost its antigenic properties but before flocculation has occurred.

Preparations of antigenic material that have been fractionated from 1% aqueous solution 2 or 3 times by means of acetone according to the technique described have always been found to be free from agar or kanten according to the three colour tests elaborated by Pirie [1936]. The primary extraction product (10 mg.) yields a reddish-purple colour whereas the same quantity of the phospholipin-free complex (to be mentioned later) gives a purple colour only with the carbazole reagent. Under the same conditions agar gave rise to a very dark blue coloration in a much shorter time. It should be mentioned in connexion with the carbazole test for agar that glucosamine and chondrosamine also give greenish-blue colorations which, however, are much less intense than the purer green colour given by an equal weight of agar or its derivative. The diphenylamine reagent gives rise to no appreciable colour with the antigenic material (10 mg.) in a time of heating which produces a strong green colour with about 1 mg. of agar. It has already been stated [Morgan, 1937] that anti-agar horse immune-serum gives no precipitin reaction with the antigenic material. The results of these experiments indicate that the antigen preparations can contain at the most only an insignificant quantity of agar or kanten.

The general chemical properties of the primary extraction product have already been described [Morgan, 1937] together with a preliminary account of the nature of the acid hydrolysis products. This part of the work will now be described in greater detail.

Hydrolysis of the primary extraction product

The antigenic material (3·0 g.) was shaken with water (150 ml.) until the material was redissolved. The material, which dissolved completely, could not be thrown out of solution by centrifuging at normal speeds (2000–3000 r.p.m.). Acetic acid was added to make a 0·1 *N* solution and the colloidal solution was heated at 95–98° on a boiling water bath for 4 hr. After 30 min. some insoluble material had separated and the opalescent solution subsequently became clear after a further 2 hr. heating. The solution was cooled and kept at 0° overnight after which time a thin film of white semi-solid fatty material had separated as a surface layer on the colourless aqueous solution and a heavy white precipitate was present in the bottom of the flask.

The hydrolysis products were repeatedly extracted with freshly distilled ether until no further material could be removed. The ether extract was dried over anhydrous Na_2SO_4, filtered and immediately evaporated to dryness *in vacuo*. The ether-soluble substance (Fraction I, 0·315 g.) represented 10·5% of the total antigen. The material was redissolved in pentane and filtered before the final process of evaporation and weighing was carried out.

The aqueous solution, after warming to 40° under reduced pressure to remove ether, was cooled to 0° and the precipitate collected by centrifuging, washed twice with 5 ml. of ice-cold distilled water and dried *in vacuo* over $CaCl_2$. The material (Fraction II, 0·65 g.) was a buff-coloured powder and represented 21·7% of the total antigen. In other experiments somewhat lower figures were obtained for this fraction.

The aqueous solution and washings were evaporated to about 25 ml., treated with 400 ml. of absolute alcohol and kept at 0° for about 18 hr. The precipitated polysaccharide was collected by centrifuging, washed several times with 95%

alcohol, then with absolute alcohol and finally dried *in vacuo*. The material was dissolved in 10 parts of water, a small insoluble residue was removed and the polysaccharide was again precipitated by the addition of 10 vol. of absolute alcohol, washed with 95% and absolute alcohol and dried *in vacuo*. The white amorphous polysaccharide material (Fraction III, 1·52 g.) represented 50·5% of the total antigenic material hydrolysed.

The alcoholic supernatant solution together with the washings were evaporated to dryness *in vacuo*; the material (Fraction IV) weighed 0·46 g. and represented about 15% of the antigenic complex. The material gives a test for arginine but does not appear to contain any appreciable amount of Fraction II (polypeptide-like component). The material reduces Fehling's solution only after acid hydrolysis.

The results obtained from many similar hydrolysis experiments, but using smaller quantities of antigen, suggest that the following values represent the ranges of variation to be expected for each component. Fraction I, ether-soluble material, 9–12%; Fraction II, insoluble in dilute acid, 17–20%; Fraction III, 50–55%.

The difficulties of separating such complex substances quantitatively and in a pure condition are well known and it is not claimed that the figures given are as accurate as those obtained in estimations involving an accepted quantitative procedure. The method of isolation, however, once chosen, was adhered to throughout the work and the purity of the components finally isolated was checked by N or P determinations. Losses due to unavoidable decomposition and partial solubility must be expected and the figures given almost certainly represent minimum values. The values given for the amount of polysaccharide are known to be 10–20% too low. This correction is based on the results of experiments made to determine the percentage recovery of small quantities of the pure polysaccharide when it is isolated under conditions similar to those existing after the acid hydrolysis of the antigenic complex.

Examination of the ether-soluble component (Fraction I) of the antigenic complex

Saponification. A portion (0·178 g.) of the ether-soluble material (Fraction I) was dissolved in pentane (8–10 ml.) and shaken thoroughly with 5 ml. of 5% Na_2CO_3 and subsequently with three 5 ml. amounts of distilled water. The combined aqueous extracts were acidified with acetic acid and extracted with freshly distilled ether, the ether was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. About 20 mg. of a white crystalline organic substance were obtained, representing 11% of Fraction I; although this fraction has not been examined in detail the evidence so far available indicates that it most probably consists of mixed fatty acids.

The pentane solution after treatment with aqueous Na_2CO_3 and extraction with water as described above was dried, filtered and evaporated to dryness. A white waxy solid (137 mg.) was obtained; $[\alpha]_{5461} + 12° \pm 2°$ (*c*, 1 in pentane). Total P, 3·9%; inorganic P, nil. N, 1·8% (Kjeldahl). Average analytical figures given in the literature for the phosphatide, kephalin; P, 3·7–4·0%; N, 1·7–1·8%. The P : N ratio is thus in both cases almost exactly unity, which indicates that the fatty material under examination probably belongs to the monoaminophospholipin group of substances. The phosphatide is only sparingly soluble in absolute alcohol and in this property it resembles kephalin rather than lecithin.

The phosphatide material was dissolved in 3 ml. of methyl alcohol which contained 140 mg. of KOH and was saponified by heating on a boiling water bath for 1 hr. under reflux. The cooled solution was diluted with 9 ml. of distilled water and was thoroughly extracted 4 times with 25 ml. amounts of ether; the ether was then extracted 3 times with 3 ml. quantities of 25% methyl alcohol, once with 1 ml. of dilute NaOH and again with 3 portions of 25% methyl alcohol.

The ether, after extraction, contained the unsaponifiable matter present in the original fatty material; this amounted to only 5 mg. and represented approximately 3% of the original ether-soluble fraction.

The main 25% methyl alcoholic solution and the combined 25% methyl alcoholic extracts were freed from methyl alcohol by heating *in vacuo*, the aqueous solution was acidified with dilute H_2SO_4 until just blue to Congo red (*p*H 4) and it was thoroughly extracted with pentane to remove free fatty acids. The pentane solution was dried over anhydrous Na_2SO_4, evaporated to dryness and sealed in an ampoule *in vacuo*. The white, semi-solid and partly crystalline material so obtained consisted of the mixed fatty acids derived from the phosphatide, weighed 89 mg. and represented 65% of the total pentane-soluble hydrolysis products.

Separation of the fatty acids. A separation of the mixed fatty acids was carried out by the method of Twitchell [1921] in which the saturated and unsaturated acids are isolated according to the solubility of their lead salts in 95% alcohol. The total material (89 mg.) was dissolved 95% alcohol (1 ml.), heated to boiling point and treated with 1 ml. of a boiling 95% alcoholic solution of lead acetate. The mixed solutions were allowed to cool slowly and to remain at room temperature for at least 2 hr.

The isolation of palmitic acid. The crystalline lead salt that separated was filtered off, washed with 95% alcohol and absolute alcohol and finally dried *in vacuo*. The material, which weighed 51 mg. and melted sharply at 103–104° (corr.), was suspended in water, treated with dilute HCl to remove Pb and thoroughly extracted with ether. The ether extract was then washed with water until the washings were neutral, dried over anhydrous Na_2SO_4 and evaporated to dryness whereby 25 mg. of a fatty acid, M.P. 49–54°, were obtained. Recrystallization of the acid from acetone at −80° finally gave a substance which melted at 58–60° (corr.) and showed no depression of M.P. when mixed with pure palmitic acid (M.P. 59–61°) whereas a specimen mixed with stearic acid melted several degrees lower. A portion of the acid was dissolved in 5 ml. of freshly distilled ether and treated with an ethereal solution of diazomethane until a permanent yellow colour persisted. After 10 min. the solution was evaporated to dryness *in vacuo* and the crystalline residue was recrystallized from ether-pentane. The methyl ester crystallized in needles, M.P. 26–29°, and a mixed M.P. of the ester with the corresponding ester of palmitic acid (M.P. 28–31°) melted at nearly the same temperature (27–29°). Palmitic acid is therefore considered to be a component of the phospholipin present in the antigenic complex.

Isolation of oleic acid. The Pb salt soluble in cold 95% alcohol, together with the alcoholic washings obtained during the isolation of the Pb salt of palmitic acid, were immediately decomposed by treatment with dilute HCl and the free fatty acid recovered by extraction with ether. The ethereal solution after being washed free from mineral acid with water was dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*; the material was stored in a sealed ampoule containing CO_2. The fatty acid (58 mg.) was obtained as a pale yellow oil.

The material was dissolved in absolute ether (2–3 ml.) and after cooling to $-10°$ was treated with an excess of bromine which was also dissolved in dry ether. The mixture was shaken and kept at $-10°$ for 2 hr. but the separation of an insoluble hexabromide [Hazura, 1887] did not occur. A control experiment in which 50 mg. of linolenic acid were similarly treated gave a good yield of the insoluble hexabromide. The ether was removed *in vacuo* and was replaced by pentane but the material was again found to be completely soluble. A sample of linoleic acid when treated in this manner gave a good yield of the pentane insoluble tetrabromide. The results of these experiments indicated that linoleic acid and linolenic acid were not present in appreciable quantities in the fatty material under examination. The liquid dibromide of oleic acid is readily soluble in ether or pentane and cannot be isolated by this method.

The pentane solution which presumably contained a soluble bromo derivative was freed from bromine by heating under reflux for 1 hr. with 100 mg. of zinc powder and 3 drops of glacial acetic acid. The solution was cooled and, together with the insoluble residue in the flask, was thoroughly extracted with dilute HCl in presence of ether. The ethereal extracts were dried over Na_2SO_4 and evaporated to dryness *in vacuo* when about 37 mg. of a pale yellow oil were obtained. The oil was dissolved in the minimum amount of ether in an atmosphere of CO_2 and was sublimed at 0·005–0·01 mm. Three fractions were collected: (1) bath temperature 30–70°, 3 mg.; (2) bath temperature 70–130°, 28 mg.; (3) bath temperature 130–160°, about 5 mg. There was only a trace of residue after sublimation. The middle fraction was dissolved in light petroleum (B.P. 60–80°) and heated under reflux with thionyl chloride until evolution of HCl had ceased; during this process moisture was rigorously excluded. The petroleum solution of the acid chloride of the unsaturated fatty acid was evaporated to dryness *in vacuo*, the petroleum replaced by 10–15 ml. of freshly distilled ether and an ethereal solution of an excess of *p*-phenylaniline was added. After keeping the solution at room temperature for 10 min. the excess of reagent was removed by treatment with dilute HCl. The ethereal solution of the derivative was then washed with dilute NaOH, finally with water and, after drying over anhydrous Na_2SO_4, the *p*-phenylanilide of the fatty acid was obtained crystalline by removing the solvent *in vacuo*. After one recrystallization from methyl alcohol at low temperature, the derivative melted at 120–121°. Yield 30 mg. The *p*-phenylanilide of oleic acid melts at 120–121° [Kimura & Nihayashi, 1935] and a mixed M.P. of this substance with the *p*-phenylanilide of the unknown fatty acid melted at the same temperature. The unsaturated fatty acid present in the phospholipin complex appears to be oleic acid.

The presence of other organic acids. The aqueous solution after extraction with pentane in order to remove the fatty acids liberated during the saponification of the phosphatide was extracted with ether to isolate lower aliphatic acids or dicarboxylic acids such as succinic acid, should these be present. The ether extract, however, yielded only a trace of material on evaporation to dryness from which it appears that simple aliphatic and other acids are not present in the hydrolysis products.

The isolation of glycerophosphoric acid. Examination of the acidified aqueous solution derived from the alkaline hydrolysis products of the phosphatide, after it had been thoroughly extracted with pentane and ether to remove the saturated and unsaturated fatty acids, showed that about 2·5 mg. of organically bound phosphate were present. Inorganic phosphate was absent. A small portion of the solution was concentrated to dryness and heated in a tube with a small piece of filter paper previously moistened with a freshly prepared 2% alcoholic solution

of piperidine which contained a drop of a solution of sodium nitroprusside. The filter paper developed a bright blue colour which on subsequent treatment with dilute alkali immediately changed to a reddish-purple. According to Feigl & Frehden [1937] this test is specific for glycerol. The main part of the solution was therefore exactly neutralized by the cautious addition of KOH; it was evaporated to about 0·5 ml., 20 vol. of absolute alcohol were added and the solution was kept at 0° for some hours in order to throw out of solution as much K_2SO_4 as possible. The solution was filtered from K_2SO_4, concentrated to a small volume and the resulting syrup taken up in methyl alcohol and treated with methyl alcoholic barium acetate and a drop of baryta solution until precipitation of the Ba salt was complete. The solution was kept at 0° overnight, after which time the supernatant fluid was found to be almost free from P. The Ba salt was filtered off, washed with a small volume of 90% methyl alcohol to remove barium acetate and with absolute alcohol and dried *in vacuo*. The Ba salt (25 mg.) was dissolved in the minimum quantity of boiling distilled water and filtered from traces of insoluble matter. The colourless filtrate was allowed to cool slowly and was kept at 0° overnight when a Ba salt separated from solution. The substance was filtered off, washed with 90% alcohol and finally with absolute alcohol and dried *in vacuo*. A solution of the Ba salt, after heating at 100° for 20 min. with 2*N* HCl failed to reduce Fehling's solution, from which it was inferred that hexose- or pentose-phosphoric acids were absent. Analysis of the Ba salt gave: Total P, 9·1; inorganic P, nil. Ba, 39·6%. Ratio, Ba : P, 1 : 0·229. Ba glycerophosphate $C_3H_7O_6PBa, H_2O$ requires P, 9·5. Ba, 42·1%. Ratio, Ba : P, 1 : 0·225. The analytical figures indicate that the Ba salt was not quite pure but this cannot be considered surprising owing to the difficulty of effecting a thorough purification when only a few mg. of material are available.

In the presence of $BaNO_3$ the Ba glycerophosphate which had been isolated failed to form a complex salt $2C_3H_7O_6PBa\text{-}Ba(NO_3)_2$ as described by Karrer & Salomon [1926] for the isolation and identification of Ba-β-glycerophosphate. A control experiment, in which only 5 mg. of an authentic specimen of the β-salt were employed, gave rise to a crystalline mass of the $BaNO_3$ complex within a few minutes of mixing the two solutions. On the other hand a similar experiment with twice this quantity of Ba-α-glycerophosphate failed to deposit an insoluble complex when treated with $BaNO_3$ solution. These experiments indicate that the glycerophosphoric acid present in the phospholipin fraction is essentially in the α-form.

The examination of the acid insoluble component (Fraction II) of the antigenic complex

Properties

Earlier work [Morgan, 1937; 1938, 2] has already shown that the material which remains insoluble in dilute acetic acid after the products of acid hydrolysis have been thoroughly extracted with ether or pentane is most probably polypeptide in nature.

The material is an acidic amorphous substance which yields a clear viscous solution in dilute alkali and in Na_2HPO_4 buffer (pH 8·4). The substance is almost completely insoluble in dilute acid, but is soluble in 30% H_2SO_4. The analytical figures for different preparations vary slightly but representative values are: C, 44; H, 6·7; N, 11·5% $[\alpha]_{5461} - 48°$ (c, 0·5 in 0·2*N* NaOH). P accounts for less than 0·2% of a carefully purified specimen from which it appears that P is not a normal constituent of the polypeptide component.

The polypeptide gives a positive biuret test and a strong test for arginine (Sakaguchi reaction). After acid hydrolysis, however, the biuret test becomes negative and the hydrolysis products give an intense yellowish-red coloration with diazobenzenesulphonic acid in alkaline solution, a positive α-nitroso-β-naphthol test for tyrosine and a strong ninhydrin reaction. One preparation which had been purified from small amounts of the other components of the antigenic complex induced in rabbits traces only of precipitins for the polypeptide itself, whereas no immune-body against the specific polysaccharide, the whole antigenic complex or *Bact. dysenteriae* (Shiga) could be demonstrated by precipitation or agglutination tests respectively. The material dissolved in phosphate buffer at *p*H 8·0 is not coagulated on boiling. An alkaline solution of the polypeptide dialyses through a cellophane membrane only very slowly. In one experiment, in which the polypeptide component was dissolved in phosphate buffer at *p*H 8·0 and dialysed against the same buffer at 0°, 25% of the total polypeptide diffused during 7 days. After 5 days more 33% of the polypeptide had diffused and at the end of 18 days 42% had passed through the membrane. The experiment was then discontinued. The diffusate and the substance remaining in the bag gave strong colour reactions for arginine.

The acid hydrolysis of the polypeptide. Hydrolysis with 2*N* HCl at 100° is difficult owing to the low solubility of the polypeptide in dilute mineral acid. Furthermore, the prolonged hydrolysis that becomes necessary under these conditions induces considerable discoloration. The hydrolysis is therefore best carried out in 10*N* formic acid which contains 3·6% HCl. The acid mixture readily dissolves the polypeptide and the solution develops only a very slight yellow colour during the hydrolysis. The *p*H of the acid mixture is approximately equivalent to that of *N* HCl. With this acid mixture it is advisable to carry out the heating in small sealed tubes completely immersed in a boiling water bath. Under these conditions the hydrolysis, as measured by increase in free amino-groups (Van Slyke), is complete in 2 hr. and the amino-N increases during the hydrolysis from less than 10% to about 50% of the total N. In all determinations the formic-HCl mixture was exactly neutralized before the Van Slyke determination was made and numerous blank experiments were included. According to the colorimetric method of Folin & Ciocalteu [1927] for the estimation of tyrosine, the polypeptide contains about 8·0% of this amino-acid and at least 5·5% of arginine, as determined by the quantitative procedure of Weber [1930], is also present in the hydrolysis products.

Tryptic digestion of the polypeptide. The polypeptide is readily attacked by trypsin in alkaline solution (*p*H 8·5) and subsequent acidification of the solution no longer precipitates unchanged amorphous polypeptide. The polypeptide (N, 11·6%) was dissolved in Na_2HPO_4 buffer (*p*H 8·4) to yield a solution that contained 13 mg. per ml. and 2 mg. of a commercial trypsin preparation were added to each ml. After keeping at 37° for 12 hr. a further equal quantity of trypsin was added and the incubation continued for 12 hr. more. At the end of this time the polypeptide was not thrown out of solution when the mixture was acidified whereas from the control solutions the polypeptide was precipitated unchanged. A determination of the free NH_2 groups (Van Slyke) showed that after tryptic hydrolysis 46% of the total N was in this form.

A more detailed study of the polypeptide-like component (Fraction II) is being undertaken.

The polysaccharide component (Fraction III) of the antigenic complex

This fraction has already been described in some detail [Morgan, 1936; 1938, 1] and has been shown to consist, in part at least, of *d*-galactose and *l*-rhamnose. The presence of an *N*-acetylhexosamine was also detected and according to a colorimetric method [Morgan & Elson, 1934] was estimated to represent between 20 and 25% of the polysaccharide complex. Several attempts to isolate and identify the hexosamine from the acid hydrolyses products of the polysaccharide failed, but since that time a method has been elaborated [Jolles & Morgan, unpublished results] for the isolation of small quantities of glucosamine and chondrosamine and it is now believed that the hexosamine is glucosamine. The polysaccharide that is isolated after acetic acid hydrolysis of the antigenic complex contains about 0·5% of P in organic combination. Full details of this part of the work will be given in a later paper.

Degradation of the antigenic complex

It would appear from the results of the experiments so far described that the antigenic material, that has been termed the primary extraction product, is built up from not less than three components: (1) the specific polysaccharide, (2) the polypeptide-like body and (3) a phospholipin.

In an attempt to obtain evidence which would indicate whether all of these components are necessary for the manifestation of antigenicity, methods have been employed by means of which either the phospholipin or the polypeptide can be eliminated from the antigenic complex as desired. As a result it has been possible to degrade the antigenic material in a stepwise manner and investigate the chemical and immunological properties of the resulting substances [Morgan & Partridge, 1939, 2]. Thus, by the action of trypsin on the antigenic material a product can be obtained which is almost free from polypeptide. Formamide treatment of the resulting polysaccharide-phospholipin complex eliminates the latter component and yields the specific polysaccharide. On the other hand, solution of the antigen in pure formamide with subsequent fractionation by means of alcohol eliminates the phospholipin and a complex is obtained which consists of polysaccharide and polypeptide [Morgan & Partridge, 1939, 1].

The dissociating action of formamide

Partial dissociation. A typical procedure adopted for the removal of phospholipin from the natural antigen was as follows. The antigenic material (2 g.) was dissolved in 120 ml. of anhydrous, neutral formamide (M.P. 1–2°)[1] and kept at room temperature for 1 hr. The formamide solution, which was somewhat opalescent, was centrifuged to remove traces of insoluble material and the supernatant fluid was treated with 2 vol. of ice-cold absolute alcohol. A small precipitate formed and was removed and the solution was treated with a further volume (120 ml.) of alcohol. This concentration of alcohol (75% by volume) precipitated from solution the greater part of the antigenic complex which was immediately collected by centrifuging, resuspended in water, dialysed and evaporated to dryness *in vacuo* below 0°. The 75% alcoholic supernatant fluid was found to contain practically the whole of the phospholipin component together with small amounts of phospholipin-free antigen and polysaccharide. Experience has shown that it is advisable to repeat the fractionation from

[1] Supplied by Dr Fränkel, Treforest Estate, near Cardiff.

formamide solution with the material that has been precipitated between 66 and 75 % alcohol concentration, as described above, in order to eliminate completely the last traces of the phospholipin.

An attempt was made to fractionate a preparation of phospholipin-free material. A 1 % aqueous colloidal solution of the material (50 ml.) was cooled to 0° and was treated with acetone. At the 60 % level a precipitate formed and was removed. Acetone was added until the solution contained 70 % when a further fraction was collected. The two main fractions were again fractionated from 1 % aqueous solution by acetone at 60 and 70 % levels and the first and last fractions were examined. The material (60 mg.) precipitated with 60 % acetone was found to yield 20 % of the polypeptide component and 57 % of polysaccharide after acid hydrolysis whereas the most soluble material (280 mg.) thrown out at 70 % acetone level contained 19 % polypeptide and 58 % polysaccharide. The original phospholipin-free material used in this experiment yielded, after acid hydrolysis, 20 % polypeptide and 61 % of polysaccharide. It would appear, therefore, that the original complex contained a slight excess of uncombined polysaccharide but was otherwise fairly homogeneous in character. A phospholipin-free complex of this nature, however, will only be obtained if the so-called natural antigen is exposed to cold, neutral formamide for a short time. The prolonged action of a large excess of neutral or slightly acid formamide will give rise to a main fraction that is not only free from phospholipin but is also seriously diminished in its content of polysaccharide. On the other hand treatment of the original antigenic complex with insufficient neutral formamide will restrict the dissociation of the components and give rise to a substance that will still contain a few per cent. of phospholipin. The more extensive dissociation of the antigenic material will be discussed again later.

The properties of the phospholipin-free complex. The properties of the phospholipin-free antigen are as follows. The specific rotation is somewhat higher than that shown by the so-called primary extraction product and is $+65° \pm 5°$ (*c*, 0·5 in formamide). Analytical figures for a typical specimen are: C, 42·3; H, 6·8; N, 4·4 (Dumas); ash 3·5 %. In such a preparation the acid-insoluble component (Fraction II) that can be isolated after acid hydrolysis amounts to approximately 22 % and the material gives strong biuret, α-nitroso-β-naphthol and Sakaguchi reactions. The phospholipin-free antigenic material gives an opalescent solution in water, but differs from the primary extraction product inasmuch as the addition of sufficient dilute alkali to raise the *p*H to 8 causes the solution to become quite clear whereas a solution of the original antigen remains distinctly opalescent after this treatment, although a considerable diminution in turbidity occurs. The addition of dilute acid to a solution of either preparation to lower the acidity to *p*H 1 causes only a slight decrease in opalescence. The phospholipin-free material is strongly antigenic and 3 doses, each of 0·01 mg. given intravenously to rabbits, induced the formation of potent immune-sera in each of the 6 animals immunized. In some sera an agglutination titre of 1 : 1280 was recorded and some of the immune-sera showed specific *Bact. dysenteriae* (Shiga) haemolytic antibody at a dilution of 1 : 2000. All the immune-sera gave immediate and heavy precipitates on addition of the homologous antigen, the primary extraction product (original antigenic material) or the specific polysaccharide. Although the phospholipin component seems, on the basis of these immunization experiments, to be unnecessary for the manifestation of antigenicity it is impossible to be certain that it has no immunological function. For example, it is conceivable that the natural antigen with its associated phospholipin component is a more potent antigen weight for

weight than the simpler polysaccharide-polypeptide complex. Unless, however, the difference in antigenic capacity of these two complexes is large there is no simple method available by means of which it is possible to obtain evidence in support of this suggestion.

A 1 % aqueous solution of the phospholipin-free antigen was centrifuged for 1 hr. at 14,000 r.p.m. in an Ecco-Blitz centrifuge in order to determine whether the material thrown out of solution or the material remaining in the supernatant fluid was antigenic. The greater part of the material was thrown down as a whitish gel whereas the supernatant fluid became quite clear and colourless. The precipitate was made up to the original volume in saline and tested, together with the supernatant fluid, for antigenicity. An excellent response was given by two rabbits that each received 3 doses of 0·05 mg. of the centrifuged deposit whereas no appreciable antibody production could be demonstrated in the sera of two other animals which received material that remained in the supernatant fluid. It should be mentioned, however, that although the supernatant fluid gave a positive Molisch reaction the amount of material contained therein represented only a few per cent. of the total antigenic material used in the experiment.

Complete dissociation. Repeated formamide treatment of the natural antigen, especially when the formamide contains 2–3 % of formic acid, carries the dissociation of the antigenic complex beyond the simple removal of the phospholipin. After the first formamide treatment the main part of the phospholipin-free material is precipitated from formamide solution by alcohol within narrower limits (66–75 % by volume) while the polypeptide component present in the precipitated material increases in amount with each formamide treatment. After two or three treatments with acid formamide the bulk of the material is precipitated from a 2–3 % solution of the preparation in formamide by 55–60 % of alcohol and this material may contain as much as 50 % of the polypeptide component (N, 11·6 %). Material of this nature possesses a much lower specific rotation ($[\alpha]_{5461}+30°$ for one preparation) than the original phospholipin-free substance and represents a partially dissociated polysaccharide-polypeptide complex that has already lost a considerable part of its polysaccharide component. Further formamide treatment would probably eliminate the polysaccharide completely but the dissociation has not been continued to this point. The polysaccharide component liberated remains in the 75 % alcoholic formamide supernatant fluid and can be recovered by dialysis to remove formamide, concentration to a small volume and fractionation from aqueous solution by alcohol. Three doses, each of 0·01 mg. of the polysaccharide prepared in this manner, fail to produce in the rabbit demonstrable "Shiga" agglutinins or haemolysins. Two examples of the products arising from the partial dissociation of the phospholipin-free antigen preparation by means of neutral and acid formamide respectively are given in Table 3. The products A and C were precipitated by 66 % alcohol from neutral and acid formamide solutions of the antigenic material. The substances B and D remained in the alcoholic formamide. In this particular experiment, after equilibrium conditions had been established in the formamide solution, 66 % alcohol precipitated a product A which contained 28 % of polypeptide and at least 56 % of polysaccharide component whereas the more soluble material, B, contained 10 % of polypeptide and a much higher proportion of the polysaccharide component. On the other hand the nature of the products arising from the dissociating action of the acid formamide indicated that a much increased dissociation of the complex had occurred, and in this experiment the material precipitated by 66 % alcohol, fraction C,

Table 3. *Products of the partial dissociation (2nd stage) of the antigenic complex in neutral and acid formamide*

		Properties of fraction			Components isolated from fractions after acid hydrolysis			
					Polysaccharide		Polypeptide	
Reagent	Fraction	$[\alpha]_{5461}$	Aqueous solution	N %	%	N %	%	N %
Neutral formamide	A (precipitate)	+40°	Strongly opalescent	4·8	56	1·6	28	8·0
	B (supernatant)	+86°	Clear	—	82	1·5	10	7·6
Acid formamide	C (precipitate)	+30°	Strongly opalescent	6·5	40	1·8	45	11·6
	D (supernatant)	+90°	Clear	2·2	76	1·7	3	4·8

contained as much as 45 % of polypeptide, whereas the more soluble material D contained only 3 % of this component.

The exact number of times the material must be treated with formamide to give a desired result must vary with the conditions employed. In most experiments a 2 % solution of the preparation in anhydrous neutral or acid formamide was kept for an hour at room temperature or several hours at 1–3° before it was fractionated with cold absolute alcohol. The properties of some of the various products arising from the original antigenic complex by the dissociating action of formamide are given in Table 4.

Table 4. *The properties of the products derived from the original antigenic complex*

				Antigenic activity in the rabbit as measured by *Bact. dysenteriae* (Shiga)*		
Material	Components	$[\alpha]_{5461}$	N %	Agglutinins	Precipitins	Haemolysins
Primary extraction product	Phospholipin Polysaccharide Polypeptide	+55 ±5° †	3·8–4·2	+ + +	+ + +	+ + +
Formamide dissociation product	Polysaccharide Polypeptide	+65 ±5° †	4·0–4·4	+ + +	+ + +	+ + +
Tryptic digest of primary extraction product	Polysaccharide Phospholipin	+60 ±5° †	2·2‡	0	0	0
Dissociation or acid hydrolysis product	Polysaccharide	+106° §	1·7	0	0	0
Do.	Polypeptide	−48° ‖	11·6	0	0	0
Do.	Phospholipin	+12° ¶	1·9	0	0	0

* After 3 doses of 0·01 mg.
† In formamide (M.P. 1–2°).
‡ This specimen contained 4 % polypeptide component.
§ In water.
‖ In 0·1 *N* NaOH.
¶ In pentane.

The action of trypsin on the antigenic material

In contrast with the result of an earlier experiment [Morgan, 1937], which suggested that the primary extraction product was antigenically active after treatment with trypsin at *p*H 8·4, later experiments, in which larger quantities of trypsin and longer digestion times have been employed, indicate that in the

earlier experiment the hydrolysis was incomplete and that some unchanged antigen remained. The removal of most of the polypeptide component from the polysaccharide-polypeptide-phospholipin complex was attained as follows. A 1 % solution (50 ml.) of the antigenic material was mixed with an equal volume of 0·5 *M* Na_2HPO_4 buffer at *p*H 8·4 and treated at 37° with four separate 25 mg. portions of a commercial trypsin preparation during 48 hr. The opalescence of the antigen solution diminished somewhat during the incubation. The solution was then dialysed against distilled water to remove sodium phosphate, centrifuged to remove insoluble material and finally, after concentration to a small volume, shaken with chloroform. The aqueous solution after centrifuging was dialysed at 0° for several days against frequent changes of distilled water, evaporated to a small volume and finally taken to dryness *in vacuo* after freezing the solution at −20°. The material thus obtained consisted of a fibrous white mass which readily dispersed in water to give an opalescent solution. The material forms a stable, colloidal solution but can be thrown down as a deposit when the solution is centrifuged for 60 min. at 4000 r.p.m. in an angle-centrifuge. The polysaccharide-phospholipin complex prepared in this manner usually contains 2–3 % of the polypeptide component and possesses only weak antigenicity. The slight immunological activity, however, is most probably due to the 2–3 % of polypeptide component which remains in the complex. The phospholipin component cannot be removed from the polysaccharide-phospholipin complex by extraction with alcohol-ether mixture or chloroform. The complex, which showed in one preparation $[\alpha]_{5461}+60\pm3°$ (*c*, 0·5 in formamide), was readily split into its two principal components by the action of 0·1 *N* acetic acid at 100° for 45 min. and by the application of the standard technique polysaccharide ($[\alpha]_{5461}+106°$ N, 1·7 %) and phospholipin (N : P, 1 : 1) components were subsequently isolated. In one specimen of polysaccharide-phospholipin hydrolysed, the polysaccharide and the phospholipin components represented 65 and 15 % respectively. The polysaccharide-phospholipin material can also be split into its components by simple solution in neutral formamide at 0° and subsequent precipitation by cold alcohol. The dissociation of the components under these conditions appears to be complete since the pure specific polysaccharide can readily be isolated after a single treatment with formamide.

The action of trypsin on the polysaccharide-polypeptide complex

A specimen (40 mg.) of antigenic material obtained by the action of neutral formamide on the primary extraction product and which yielded 60 % polysaccharide and 21 % polypeptide on acid hydrolysis was dissolved in distilled water (4 ml.) and 0·2 *M* Na_2HPO_4 (2 ml.) was added. The opalescent solution was then treated with commercial trypsin (8 mg.) dissolved in water (0·8 ml.) and incubated at 40°. In less than 1 min. the solution became quite clear but incubation was continued for 24 hr. The material was then dialysed in a small cellophane bag against frequent changes of distilled water for several days until the diffusate gave no test for inorganic phosphate thus indicating the complete removal of the buffer. The solution after dialysis was thoroughly shaken with chloroform which caused the non-diffusible portion of the trypsin preparation and a part of the polysaccharide to separate as an interfacial layer which could be subsequently removed by centrifuging. The clear aqueous supernatant fluid was then concentrated. After a further reprecipitation from aqueous solution by acetone a specimen of polysaccharide ($[\alpha]_{5461}+98°$; N, 1·5 %) was obtained.

The reversibility of the formamide dissociation of the antigenic polysaccharide-polypeptide complex

The polypeptide component prepared by acetic acid (1 %) hydrolysis of the polysaccharide-polypeptide complex and subsequently purified by repeated precipitation from alkaline solution by dilute HCl and trituration with absolute alcohol is only slightly soluble in formamide or in a solution of polysaccharide in formamide. On the other hand a preparation of the polypeptide when freshly precipitated from solution in alkali by acid is still insoluble in formamide but dissolves readily in formamide in the presence of the specific polysaccharide. The utilization of this property has enabled an artificial polysaccharide-polypeptide complex to be prepared. A specimen (60 mg.) of polysaccharide prepared by formamide dissociation of the natural polysaccharide-polypeptide complex was dissolved in 2·5 ml. formamide (M.P. +2°). The clear viscous solution was added to a carefully purified sample (40 mg.) of polypeptide which was in the form of a water-moist centrifuged deposit. The polypeptide dissolved immediately on stirring the mixture and a clear viscous solution was obtained. This was kept at room temperature for 2 hr., diluted with an equal quantity of formamide and kept at 35° for 2 hr. longer, in order to allow the system to attain equilibrium. The clear solution was then cooled to 0° and absolute alcohol (also at 0°) added to yield an overall concentration of 75 %. A precipitate formed and was collected by centrifuging. This deposit was immediately taken up in 10 ml. of distilled water and it remained as a stable opalescent suspension during several days' dialysis at 0° against frequent changes of distilled water. A small amount of material, presumably the larger particles, was removed by centrifuging at 3500 r.p.m. in an angle-centrifuge for 20 min. The artificial complex was obtained in the form of a white fibrous mass by evaporating the frozen solution *in vacuo*. A part of the preparation was heated for 1 hr. at 100° with 1 % acetic acid and under these conditions it gave rise to a flocculent precipitate of the polypeptide component in the same manner as occurs with the natural complex.

The artificial complex and the original polysaccharide and polypeptide components used in the experiments were tested for antigenicity in rabbits. A course of 6 doses, each of 0·02 mg., was given intravenously and the animals were bled after 6 days. Agglutination and precipitation tests showed that the polysaccharide and polypeptide failed to induce the formation of specific "Shiga" immune bodies, whereas the artificial complex gave rise to an immune-serum that showed agglutination to a titre of 1 in 320 with *Bact. dysenteriae* (Shiga) and gave distinct precipitation with 1 : 10,000 and 1 : 100,000 dilutions of the specific polysaccharide. The immune-serum also gave a positive precipitin reaction when mixed with an equal volume of a 1 : 1000 dilution of the polypeptide component. These interesting results immediately suggest many further investigations, and experiments are already being undertaken to determine whether the specific polysaccharides of other organisms can be combined in this manner with the "Shiga" polypeptide component to yield an antigenic complex.

Discussion

Evidence is given that the primary extraction product which has been isolated from the "smooth" strain of *Bact. dysenteriae* (Shiga) consists essentially of a homogeneous molecular complex which is able to induce the formation of antibacterial immune-bodies similar to those produced by injection of the intact

organism. This material has been used for further chemical and physical investigation.

Gentle acid hydrolysis of the antigenic material yields a polypeptide-like component, a polysaccharide and a phospholipin complex. The amounts of these constituents isolated make up altogether 80–90 % of the weight of the original antigenic material and thus indicate that a fourth component, if it exists, must be quite small in amount. The polysaccharide component is built up from *d*-galactose, *l*-rhamnose and *N*-acetylglucosamine units whereas from the phospholipin molecule oleic acid, palmitic acid and α-glycerophosphoric acid have been obtained. The nitrogenous constituent of the phospholipin has not been isolated. The polypeptide-like component is an acidic, amorphous substance containing 11·6 % N, and tyrosine (8 %) and arginine (5·5 %) have been identified by colorimetric methods as constituent amino-acids. After hydrolysis with *N*-HCl-10*N* formic acid mixture nearly 50 % of the total N of the polypeptide is in the form of free amino-groups (Van Slyke). A more detailed account of this important component of the natural antigenic complex will be given in a later paper.

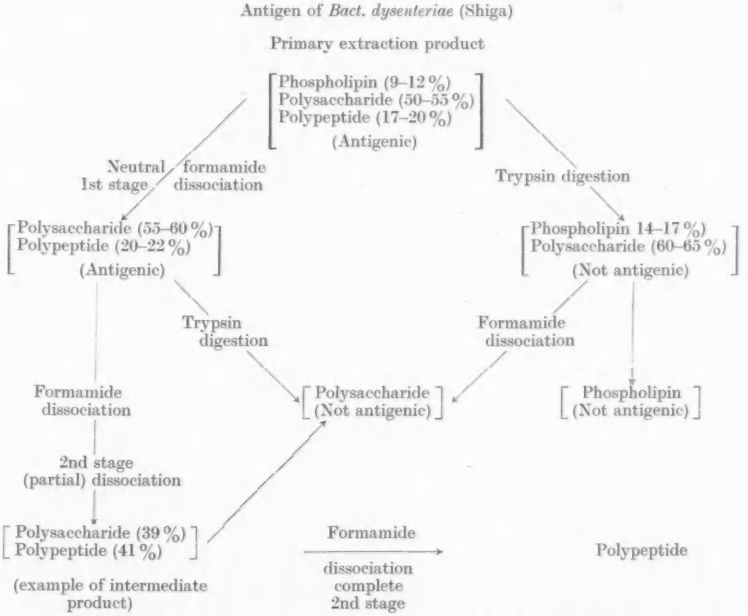
The hydrolysis experiments reveal the existence within the antigenic complex of some easily hydrolysable phosphate (about 10 % of the total P) which is presumably in organic combination but which is ultimately found in the form of inorganic phosphate in the alcoholic supernatant fluid remaining after the three principal components have been removed. This residual material (Fraction IV) may possibly contain a further component but it has not as yet been examined in any detail.

Although antigenic material has been isolated from various organisms and studied by a variety of methods there has appeared as yet no simple, systematic technique whereby it has been possible to break down the antigenic complex step by step and investigate the chemical, physical and immunological properties of the resulting degradation products. In this paper a method whereby this may be accomplished is described. The method is based on the dissociating action of the highly polar solvent, formamide. Further work will certainly modify and improve the technique to suit the nature of the material under investigation better, but it seems probable from our results that the use of highly polar organic solvents opens up a new method of attacking the problem of the constitution of certain naturally occurring hydrophobic substances of complex composition, of which bacterial antigens are but a single example. In this connexion it is of interest to recall the reported dissociation of native horse haemoglobin into molecules of half the normal molecular weight by means of aqueous formamide [Steinhardt, 1938].

The degradation of the natural antigen by means of neutral or acid formamide or trypsin into simpler complexes is shown diagrammatically in Fig. 1 where the various steps in the process and the approximate compositions of the resulting products are clearly set out. Different preparations of antigen have, under the experimental conditions described, given rise to material that differs in composition within the limits shown. This variation in composition is to be expected in view of the ease with which the components of the natural antigen are dissociated by the strongly polar solvent and it is clear that the separation of the antigenic material into products with the composition given will only be attained if the experimental conditions follow closely those described in the earlier part of this paper.

The first stage in the formamide dissociation process, the removal of phospholipin, is a relatively simple one. It appears that in formamide solution the dissociation of the phospholipin from the remainder of the complex is almost

complete and in consequence one precipitation with alcohol brings down the bulk of the material which is then found to be associated with only 1–2 % of the phospholipin in place of the original 9–12 % in the natural antigenic complex. A repetition of the process yields an antigenic material in which no phospholipin is detectable. The polypeptide component, however, is only partially dissociated from the polysaccharide in formamide solution under the conditions described, for if the phospholipin-free material is dissolved in neutral or slightly acid



Note. All polysaccharide percentages given are for material *isolated* and must be considered 10–20 % too low.

Fig. 1.

formamide and fractionated with alcohol insufficient in amount to precipitate completely the whole of the complex, the less soluble material which is first thrown out of solution contains a greater relative proportion of polypeptide than does the later material which is precipitated with increasing alcohol concentration. By the successive removal in this manner of the most soluble material—the polysaccharide—a polysaccharide-polypeptide complex may be obtained which contains as much as 50 % of the later component. Material that contains as little as 2–3 % of polypeptide has been isolated from the more soluble fraction and preparations containing 5–10 % of this component have been tested and found to be antigenic. The polysaccharide remaining in the final alcoholic formamide possesses properties very similar to that of the polysaccharide isolated by direct treatment of the micro-organisms with hot acetic acid [Morgan, 1936] except that it dissolves less readily in water, gives rise to a

solution of high viscosity and contains about 0·5% of phosphate in organic combination. The polysaccharide isolated by either method, however, is not antigenic. In view of the mild conditions of treatment involved in the alcoholic formamide fractionation process it is unlikely that the polysaccharide has been altered chemically during its dissociation from the polypeptide component. It appears probable, therefore, that the loss of antigenicity of the complex is associated in some manner with the loss of the polypeptide and not with any chemical change in the polysaccharide molecule. An essential function of the polypeptide, which is itself but weakly antigenic, may be to create a mechanism whereby intermolecular association is able to occur that will ultimately give rise to a large hydrophobic colloidal particle. A complex of this nature which is not readily eliminated by the animal body would presumably possess antigenic properties. A mechanism which it is thought might explain the dissociating action of formamide has been suggested by Steinhardt [1938] who considers it likely that formamide functions as a dissociating agent for proteins because it possesses an amide bond similar to that which occurs repeatedly as the peptide link in proteins and which is responsible for a considerable measure of polar association between the polypeptide chains. Steinhardt suggests that the formamide becomes involved in the competition for the peptide bonds of adjacent chains and thus weakens or eliminates completely the tendency of the bonds to associate. It is of interest to recall that the dialysis of a clear solution of the antigenic material in formamide gives rise to strongly opalescent aqueous solutions, presumably owing in part to the reaggregation of the dissociated components since the product that can be subsequently isolated is similar in general chemical and immunological properties to the original material. A number of experiments have been carried out with the object of ascertaining whether it is possible to reconstitute the original antigenic complex from the antigenically inactive components. The experiments have not been described in any detail owing to their preliminary nature but it has been observed that when the essential components of the antigenic complex—the polysaccharide and polypeptide—are mixed together in formamide solution and the resulting complex is subsequently isolated by an appropriate technique, then this complex has definite antigenic properties and gives rise to specific *Bact. dysenteriae* (Shiga) agglutinins and precipitins. From the immunological standpoint this result indicates that after the complete dissociation of the components of the system, their subsequent re-aggregation, which results from the removal of the dissociating solvent, occurs in a regular manner.

The method used for the extraction of the antigenic material from the bacterial cell suggests that the primary extraction product is in reality a degraded form of the antigenic complex as it exists in the living micro-organism. This suggestion would offer an explanation for the observed fact that commercial trypsin does not attack the antigen when it is present in the intact bacterial cell whereas preparations of the antigenic complex isolated by means of diethyleneglycol are slowly attacked. If one may judge from the results obtained by the use of formamide, extraction of the antigenic material with diethyleneglycol will induce some measure of dissociation which will result in the loss of part of the original antigenic complex. Diethyleneglycol, however, with its low dielectric constant would almost certainly be less active as a dissociating medium than the strongly polar formamide which possesses a remarkably high dielectric constant. In any event, the antigenic material after isolation is slowly attacked by trypsin and is thereby almost completely deprived of its polypeptide-like component. Furthermore, the phospholipin-free antigenic material, which represents a

further stage in the degradation of the antigenic complex, is quite readily attacked by the enzyme preparation with destruction of the polypeptide component and the loss of antigenic properties. A similar difficulty in removing a polypeptide-like component which is bound to polysaccharide has been reported recently by Hewitt [1939] who attempted to isolate the polysaccharide components of seroglycoid and globoglycoid by means of both peptic and tryptic hydrolysis. His results show that even after one treatment with pepsin followed by three treatments with trypsin the carbohydrate represented only about 50 % of the total carbohydrate-polypeptide complex. It would be of interest to know whether these glycoproteins can be separated more readily into their polysaccharide and protein components by means of formamide.

There is additional evidence that the antigenic complex is changed in some manner during the extraction process. The original antigenic material shows an appreciable solubility in anhydrous diethyleneglycol, whereas after isolation from this solvent, a process that can be accomplished by simple dialysis and evaporation at low temperature, the material no longer shows any definite solubility in diethyleneglycol. It seems probable, therefore, that the insolubility of the isolated material is due to loss of a part of the complex as a result of the dissociating action of the diethyleneglycol.

It has been observed that the opalescence of an aqueous solution of the polysaccharide-polypeptide complex in phosphate buffer at *p*H 8·4 disappears completely a few seconds after the addition of trypsin. At this stage the polypeptide is apparently liberated from its union with the polysaccharide but it is not split into its constituent amino-acids as can be readily shown by acidifying the reaction mixture, which results in the immediate precipitation of the insoluble polypeptide component. The rapid dissociation of the polypeptide from its combination with the polysaccharide recalls the recent observations of Pope [1938; 1939] on the disaggregating action of enzymes on antitoxic pseudoglobulin, whereby the complex molecule is split into two different components with no demonstrable degradation into the constituent amino-acids. This interesting phenomenon is being further investigated.

By the action of neutral formamide it has been possible to remove the phospholipin component from the primary extraction product and obtain a substance that consists essentially of a complex of polysaccharide and a polypeptide-like material. This substance is strongly antigenic and gives rise to specific "Shiga" heterophile immune-body as well as "Shiga" agglutinins and precipitins. In view of the general belief that phospholipins play an important part in the antigenic structures which lead to the production of heterophile haemolytic antibody it is of considerable interest to find that the antigenic complex after treatment with neutral formamide, which removes the phospholipin component, remains strongly antigenic and induces the formation of "Shiga" haemolytic immune-body qualitatively identical with that produced by means of the intact micro-organism. This result, however, is in complete agreement with the evidence brought forward by Meyer & Morgan [1935] and Meyer [1938], that the specific polysaccharide of *Bact. dysenteriae* (Shiga) possesses the property of neutralizing the haemolytic action of "Shiga" heterophile antibody on sheep red cells, in addition to its property of combining with the homologous antibacterial immune-substance as indicated in agglutination, precipitation and complement-fixation tests. It has also been shown that the polysaccharide-phospholipin complex which arises from the prolonged action of trypsin on the antigenic material is most probably incapable, when completely free from the polypeptide component, of inducing in the rabbit the formation of "Shiga"

heterophile immune-body. These two pieces of evidence taken together indicate that the phospholipin component plays no significant part in the production or neutralization of "Shiga" haemolytic antibody.

An attempt has been made to find out whether the antigenic material isolated by means of trichloroacetic acid according to the technique of Boivin *et al.* [1935] is similar in general properties and behaviour to the material obtained by extraction with diethyleneglycol. The results are inconclusive, for although the polysaccharide component (54%) and pentane-soluble phospholipin (6%) appear to be identical, the polypeptide could not be isolated by the usual technique. The preparation was found to contain other nitrogenous material and it has not as yet been possible to separate a polypeptide component identical with our own. The antigenic material extracted by means of trichloroacetic acid, however, gave rise to a phospholipin-free product on treatment with formamide which was found to be antigenic when tested in the rabbit.

Although it is perhaps beyond the scope of the present communication to suggest the nature of the intermolecular forces that bind together the various component molecules which make up the natural antigenic complex, it would appear from the results that have been obtained that these bonds are not of the covalent type and that in consequence stereochemistry of the linkages and constant molecular composition for the antigenic complex are not to be expected. It must remain, however, for future work to decide whether the association of the various component molecules of the antigenic complex is due to polar forces alone, to the "hydrogen" or to the "hydroxyl" bond or to some other as yet unknown mechanism.

As a result of some earlier experiments in which antigenic material prepared by three different methods was examined, the idea that perhaps a bacterial antigen was not a chemical compound of fixed composition gradually developed. It appeared conceivable that a bacterial antigen as it existed in the intact bacterial cell was not a single chemical compound of rigid composition but consisted of a labile molecular aggregate possessing an essential component—such as a polysaccharide—of definite chemical structure and of fixed composition which determined the strict immunological specificity of the antigen, together with other loosely bound constituents which endowed the essential component with antigenic properties. It seemed probable that only part of these physically associated molecules were necessary for full antigenic activity and that certain of these constituents could, therefore, be dissociated from the labile complex during the isolation and purification of the antigen without producing more than a moderate reduction in the antigenic capacity of the active material. It was believed that further and more thorough physical disaggregation of the associated constituents would ultimately lead to complete loss of antigenicity. Some evidence in support of this conception of antigenic structure is given in this paper.

The work that has been described represents an attempt to learn something of the general structure of a bacterial antigen and the results have enabled a conception of antigenic structure to be developed. It is not believed, however, that the conclusions are in any way final. Indeed much further evidence must be collected before they can be completely accepted even in their present form, although the experimental facts given would seem sufficient to justify the temporary acceptance of the conception as a working hypothesis. It would appear as a result of similar experiments carried out with antigenic material isolated from specific strains of *Bact. typhosum* that the polysaccharide-polypeptide-phospholipin type of complex may be of fairly wide occurrence as a basic structure in certain bacterial antigens.

SUMMARY

1. The dominant antigenic complex associated with the "smooth" form of *Bact. dysenteriae* (Shiga) is shown to consist of three major components—a polysaccharide, a phospholipin and a polypeptide-like substance. Other minor components may also be present.

2. A method is described for the stepwise degradation of the antigenic complex which in part depends on the dissociating action of formamide.

3. A conception of antigenic structure is suggested which is supported by certain of the experimental results.

REFERENCES

Boivin & Mesrobeanu (1933). *C.R. Soc. Biol., Paris*, **112**, 76.
—— —— (1934). *C.R. Soc. Biol., Paris*, **115**, 304, 309.
—— —— (1937). *C.R. Soc. Biol., Paris*, **124**, 442.
—— —— & Mesrobeanu (1935). *Arch. roum. Path. exp. Microbiol.* **8**, 45.
Elson & Morgan (1933). *Biochem. J.* **127**, 1824.
Feigl & Frehden (1937). *Mikrochim. Acta*, **1**, 137.
Folin & Ciocalteu (1927). *J. biol. Chem.* **73**, 627.
Hazura (1887). *Mh. Chem.* **8**, 463, 472.
Henderson & Morgan (1938). *Brit. J. exp. Path.* **19**, 82.
Hewitt (1939). *Biochem. J.* **33**, 1496.
Karrer & Salomon (1926). *Helv. chim. Acta*, **9**, 3.
Kimura & Nihayashi (1935). *Ber. dtsch. chem. Ges.* **68**, 2028.
Mesrobeanu (1936). *Arch. roum. Path. exp. Microbiol.* **9**, 1.
—— & Calab (1936). *C.R. Soc. Biol., Paris*, **122**, 496.
Meyer (1930). *Z. Immun. Forsch.* **68**, 98.
—— (1938). *C.R. Soc. Biol., Paris*, **128**, 746.
—— & Morgan (1935). *Brit. J. exp. Path.* **16**, 476.
Miles & Pirie (1939, 1). *Brit. J. exp. Path.* **20**, 83.
—— —— (1939, 2). *Brit. J. exp. Path.* **20**, 109.
—— —— (1939, 3). *Brit. J. exp. Path.* **20**, 278.
Morgan (1936). *Biochem. J.* **30**, 909.
—— (1937). *Biochem. J.* **31**, 2003.
—— (1938, 1). *Helv. chim. Acta*, **21**, 469.
—— (1938, 2). *Chem. Ind.* **56**, 976.
—— & Elson (1934). *Biochem. J.* **28**, 988.
—— & Partridge (1939, 1). *Nature, Lond.*, **143**, 1025.
—— —— (1939, 2). *Chem. Ind.* **58**, 1020.
Pirie (1936). *Brit. J. exp. Path.* **17**, 269.
Pope (1938). *Brit. J. exp. Path.* **19**, 245.
—— (1939). *Brit. J. exp. Path.* **20**, 132.
Raistrick & Topley (1934). *Brit. J. exp. Path.* **15**, 113.
Steinhardt (1938). *J. biol. Chem.* **123**, 543.
Topley, Raistrick, Wilson, Stacey, Challinor & Clark (1937). *Lancet*, **1**, 252.
Twitchell (1921). *J. industr. Engng Chem.* **13**, 806.
Weber (1930). *J. biol. Chem.* **86**, 216.

28. THE BEHAVIOUR OF SOME URAMIDO-ACIDS IN THE NITROUS ACID METHOD FOR THE DETERMINATION OF AMINO-NITROGEN

By ALLAN GODFREY GORNALL and ANDREW HUNTER

From the Department of Pathological Chemistry, University of Toronto

(*Received 30 December 1939*)

In attempting recently to identify a substance produced by the action of arginase upon carbamido-arginine one of us [Hunter, 1938] took occasion to apply to it the well-known nitrous acid method of Van Slyke for the determination of primary amino groups. The question thereupon presented itself, whether the results obtained were consistent with the supposed constitution of the product as an α-uramido-δ-amino-acid. The behaviour of a simple amino group could of course be predicted, but a search of the literature yielded surprisingly little information upon that to be expected of a uramido group. Rohde [1918] had stated that uramido-acids yield "slightly more" than 1 atom of N after 1 hr. of interaction with nitrous acid, and had proposed upon this basis a method for their determination; but her generalization rested upon no more than two examples, of which one (the uramido-acid derived from phenylalanine) had actually given 1·25 atoms and the other (from leucine) 1·12. The only other pertinent observations we could find were those of Wada [1930] upon the α-amino-δ-uramido-acid, citrulline, which had yielded in 1·5 hr. 0·63 and in 2·5 hr. (or more) exactly 1 atom of uramido- (*plus*, of course, 1 atom of amino-) N.

This paucity of information induced us to examine the behaviour of a somewhat wider range of acids, the object in view being the purely practical one of ascertaining how far the Van Slyke method might be useful in the characterization, and possibly the quantitative determination, of such compounds. Regarded from this aspect the results were only moderately encouraging, for, as will be seen, they revealed a notable diversity of behaviour. Certain limited regularities nevertheless became evident, and it seems not impossible that a wider selection of material would have enabled us to establish these regularities as general rules. In any case, since uramido-acids have frequently been encountered in biological material, it seems worth while to put on record such data as we have collected.

The survey was facilitated by gifts of material, here gratefully acknowledged, on the one hand from Dr Henry D. Dakin, on the other from Drs Edwin J. Cohn and Thomas L. McMeekin of the Department of Physical Chemistry, Harvard Medical School. From the Harvard Laboratory we received samples of the uramido-acids derived from β-alanine, *nor*leucine and ε-aminohexoic acid; from Dr Dakin samples of the corresponding derivatives of *l*(+)-α-alanine, *l*(−)-phenylalanine, *dl*-α-aminobutyric acid, *dl*-α-amino*iso*butyric acid, *dl*-valine, *dl-nor*valine, *dl*-leucine and *dl-iso*leucine. Each of these samples had been prepared by the interaction of the corresponding amino-acid with KCNO. Descriptions of all but one will be found in the papers of Lippich [1908], Dakin [1910; 1915], Dakin & Dudley [1914], West [1918], Boyd [1933] or McMeekin *et al.* [1936]. The *nor*valine derivative does not appear to have been mentioned in the

literature; the specimen supplied to us as well as another prepared by ourselves, melted at 178–9°. Other uramido-acids included in the study were the α-uramido-δ-guanidino-, the α-uramido-δ-amino- and the α-amino-δ-uramido-derivatives of *n*-valeric acid. The first of these ("carbamido-arginine") we prepared from *l*(+)-arginine by the KCNO method [Boon & Robson, 1935], the second from the first by the action of arginase [Hunter, 1938], and the third (citrulline) from (+)-arginine in the manner described by Gornall & Hunter [1939]. With the object of checking some of our first results we prepared also additional specimens of the uramido-acids from *dl*-α-alanine, *dl*-α-aminobutyric acid, *dl*-*nor*valine, *dl*-valine and *l*-leucine.

Observations upon the 14 uramido-acids specified were supplemented by others upon urea, upon ornithine and upon argininic acid (prepared from arginine by the method of Felix & Müller [1936]). The last two were included merely to furnish appropriate corrections for the effect of a δ-amino or δ-guanidino group.

The purity of all materials studied was checked by nitrogen determinations, the specimen in hand being, if necessary, recrystallized until the result was satisfactory. For several of the analyses we have to thank Mr Stephen M. Nagy of Dr Max Bergmann's laboratory at the Rockefeller Institute, New York.

The nitrous acid method was applied in its manometric form [Van Slyke, 1929] and always under the same conditions. The volume of uramido-acid solution analysed was 5 ml., the total volume of reacting mixture 8 ml. and the volume, at which the pressure of the nitrogen was measured, 2 ml. Throughout each determination the temperature was kept as nearly as possible at 25°. Measurements were made of the nitrogen evolved not only after 3 min. (the time estimated as sufficient for the complete decomposition of an α-amino-group), but also, in separate experiments, after 30 min. and after 2·5 hr. Sometimes an additional measurement was made after 1 hr. At the end of the 3 min. interval, timed from the instant at which half of the nitrite solution had entered the reaction chamber and terminated by 1 min. of shaking, the transfer of the evolved gases to the alkaline permanganate was made with the utmost possible celerity, so as to minimize the error due to the still rather rapidly continuing evolution of nitrogen. At the later stages the speed of this evolution had been so reduced, that the effect of small errors in the time measurement had become negligible. During the 2·5 hr. interval so much gas accumulated in the reaction chamber, that the mercury tended to rise ultimately to the top of the manometer. Before this actually occurred, the gases were passed into the permanganate, the NO absorbed and the nitrogen either left in the absorption pipette or returned to the chamber. After one such preliminary absorption the reaction could be completed, and the total liberated nitrogen measured, in the usual way.

The concentration of the solutions analysed varied from 0·033 to 0·16 % and the amount of uramido-(+amino-) N in the 5 ml. sample from 0·3 to 1·6 g. These quantities were such that the nitrogen liberated in the 2·5 hr. experiment exerted (at 2 ml. volume) a pressure of not less than 190 and (except in one instance) not more than 440 mm. of mercury. Within the limits thus defined the concentration of a given substance could, it was found, be varied with little, if any, effect upon the results (see, for instance, data in Table 1 under Nos. 9B, 13A and 13B).

Each observation made on any one solution was repeated at least once, often twice or more. In many cases the result obtained with one solution or one sample of a substance was controlled by measurement on another. The degree of consistency apparent among parallel observations was in general excellent. The most considerable discrepancy was that encountered in the comparison of two different samples of α-uramido-*iso*hexoic acid (Nos. 11A and 11B).

Table 1. *Action of nitrous acid on uramido-acids, etc.*

No.	Substance	Related amino-acid	Concentration %	Atoms of nitrogen liberated in: 3 min.	30 min.	1 hr.	2·5 hr.
			Group I				
1	ε-Uramido-*n*-hexoic acid	ε-Amino-*n*-hexoic acid	0·1366	0·28	0·58	—	0·66
			0·1466	—	0·58 (0·58)	0·61	0·66 (0·66)
2 A	α-Uramidopropionic acid A	α-Alanine	0·0960	0·23	0·64	—	0·72
2 B	α-Uramidopropionic acid B	α-Alanine	0·1112	0·24 (0·24)	—		0·68 (0·70)
3	δ-Uramido-α-amino-*n*-valeric acid (citrulline)	Ornithine	0·0920	0·16	0·50	0·60	0·76
4	β-Uramidopropionic acid	β-Alanine	0·1294	0·28	0·72	—	0·78
			Group II				
5	α-Uramido-δ-guanidino-*n*-valeric acid	Arginine	0·0832	0·28	1·01	—	1·28
			0·1333	0·27 (0·28)	1·02 (1·02)	—	1·21 (1·25)
6 A	α-Uramido-*n*-butyric acid A	α-Amino-*n*-butyric acid	0·1644	0·27	1·06	—	
			0·0822	—	—	—	1·24
6 B	α-Uramido-*n*-butyric acid B	α-Amino-*n*-butyric acid	0·1037	—	—	—	1·28 (1·26)
7	α-Uramido-*n*-hexoic acid	*nor*Leucine	0·1089	0·27	1·09	1·20	1·28
8	α-Uramido-δ-amino-*n*-valeric acid	Ornithine	0·0724	0·19	0·92	—	1·30
9 A	α-Uramido-*n*-valeric acid A	*nor*Valine	0·1008	0·30	1·12	—	1·27
9 B	α-Uramido-*n*-valeric acid B	*nor*Valine	0·0995	—	—	—	1·31
			0·0498	—	—	—	1·35 (1·31)
10	α-Uramido-β-phenylpropionic acid	Phenylalanine	0·0825	0·28	1·15	—	1·42
11 A	α-Uramido-*iso*hexoic acid A	Leucine	0·0698	0·28	1·31	—	1·60
11 B	α-Uramido-*iso*hexoic acid B	Leucine	0·0818	0·31 (0·29)	—	—	1·48 (1·54)
			Group III				
12	α-Uramido-β-methyl-β-ethylpropionic acid	*iso*Leucine	0·0692	0·28	1·48	—	1·98
13 A	α-Uramido-*iso*valeric acid A	Valine	0·0328	0·30	1·55	1·81	2·00
13 B	α-Uramido-*iso*valeric acid B	Valine	0·0603	0·32 (0·31)	1·57 (1·56)	1·77 (1·79)	1·99 (1·99)
14	α-Uramido-*iso*butyric acid	α-Amino-*iso*butyric acid	0·1034	0·31	1·58	—	—
			0·0517	—	—	—	2·00
			Miscellaneous				
15	Argininic acid	—	0·1556	0·006	0·06	—	0·34
			0·1870	0·004 (0·005)	0·06 (0·06)		0·34 (0·34)
16	Ornithine monohydrochloride	—	0·0904	1·86	(2·00)	—	(2·00)
17	Urea	—	0·1656	0·12	—	—	—
			0·0552	—	0·90	—	—
			0·0344	—	—	1·34	1·76

The data secured are collected in Table 1, each figure in which is the average of 2·5 concordant observations. The order in which the uramido-compounds are arranged is that of increasing amounts of uramido-N liberated in 2·5 hr. Different preparations of any one substance are distinguished by the letters A and B. We have not thought it worth while to make note, in Table 1, of spatial configurations. For the ornithine and arginine derivatives it should be noted that the figures given represent not the total N liberated, but that part only which was estimated to have arisen from the uramido group. In the case of citrulline (No. 3) they were calculated by deducting 1 atom (representing the α-amino group) from each experimentally observed result; in the case of α-uramido-δ-guanidinovaleric acid (No. 5) by deducting at each time period the corresponding figure for argininic acid (representing nitrogen from the guanido group); in the case of α-uramido-δ-amino-valeric acid (No. 8) by deducting the nitrogen derived from the δ-amino group, this in turn being estimated by subtracting 1 atom (the α-amino-N) from the corresponding figures for ornithine (No. 16). Thus, for example, at 3 min., the actual figures were 1·16 atoms for No. 3, 0·289 for No. 5, and 1·05 for No. 8. With respect to ornithine it may be mentioned, that the time necessary for the complete liberation of its second N was found to be exactly 10 min. at 25°.

The first point evident in Table 1 is that the uramido group is decomposed by nitrous acid much less rapidly than either the α- or the ω-amino group in an amino-acid. To this extent our observations confirm those of Rohde [1918]. On the other hand it is obviously not the case that all uramido-acids liberate in 1 hr. one atom of N. The examples examined can be arranged, as shown, in three distinct, if not quite sharply delimited, groups. Those in Group I yield in the 2·5 hr. period distinctly less than 1 atom of N, those in Group II between 1·2 and 1·6 (most of them between 1·24 and 1·35) and those in Group III exactly (or almost exactly) 2. The differing behaviours of these groups are also evident at 30 min., but are either non-existent or (more probably) obscured at 3 min.

Inspection of the lists composing the different groups shows (1) that all the ω-uramido-acids examined belong to the first, (2) that with one exception the α-uramido-acids with straight chain belong to the second and (3) that, again with one exception, the α-uramido-acids with branched chains belong to the third. The exceptions—α-uramidopropionic and α-uramido-*iso*hexoic acids (Nos. 2 and 11 in Table 1)—each yield less nitrogen (or react more slowly) than other acids of analogous constitution, so that the first falls into Group I instead of Group II, and the second into Group II instead of Group III. The significance of these apparent anomalies is doubtful; but upon the second of them the following comment is perhaps pertinent. The only compounds which yield in 2·5 hr. the full 2 atoms of N are those (Nos. 12–14), in which the carbon chain branches at either the α- or the β-position. In α-uramido-*iso*hexoic acid branching occurs at a point more remote from the uramido group, and the yield of N falls. It is still, however, greater than the 1·3 atoms or so given by the straight-chain aliphatic compounds, Nos. 5–9. This suggests that the speed or completeness of the nitrous acid reaction is affected not merely by the existence, but also by the position, of a branch in the carbon chain, and, more specifically, that proximity of the branch to the uramido group is an accelerating factor. These suggestions are offered with due reserve, for the data prompting them are all too meagre.

Analogy with the amino-acids would lead one to expect that an α-uramido group would react more rapidly than one in any other position. In a general way this expectation is realized, although one departure from the rule is evident in the case of the slowly reacting α-uramidopropionic acid. The analogy with the

amino-acids extends even further. Dunn & Schmidt [1922] have shown that as the distance of the amino group from the carboxyl group increases, the speed of its reaction with nitrous acid continues to diminish. Comparison of ϵ-uramido-hexoic acid (No. 1) with β-uramidopropionic acid (No. 4) and of these with the main group of similarly constituted α-uramido-acids (Nos. 6–9) shows that, generally speaking, this is true of the uramido group also. The fact that the δ-uramido group of citrulline (No. 3) falls partly out of line (for the first hour or so its rate of decomposition is the lowest of all) is probably due to a disturbing influence exerted by its α-amino group or by the hydroxyl group into which the latter is soon converted.

A disturbing influence is in fact evident in each of the two instances in which the uramido group is accompanied by a simple amino group. A mere inspection of the numerical data suffices to show that not only in citrulline (No. 3), but also in its isomeride (No. 8), the behaviour of the uramido group diverges from the general pattern. The form of this divergence becomes clearer, if the data are plotted on a graph. The simple uramido-acids (eleven in number), and even the more complex arginine derivative (No. 5), then yield curves which, though varying in height, have all essentially the same shape. The initial rise is relatively rapid, but it soon slackens, and the later part of the curve is very flat. The curves for the amino-uramido-acids are distinctly different—their earlier parts not so steep, their later less nearly horizontal, their changes of direction accordingly less abrupt. Their whole form is in fact intermediate between that presented by any simple uramido-acid and the gradual yet prolonged upward sweep which would represent our data for urea. The validity of these comparisons depends of course upon the correctness of the assumption inherent in our calculations. The observed results have been interpreted as an effect of the amino group upon the rate of decomposition of the uramido one; the possibility of a converse or reciprocal action has been left out of the reckoning.

It was found by Van Slyke [1929] that the speed with which the α-amino groups of amino-acids react with nitrous acid is dependent not upon temperature alone, but also upon the volume of the mixture in the reaction chamber. It is probable that this is equally true of uramido groups. The results reported in this paper would then be strictly applicable only under the adopted conditions—a temperature of 25° and a volume of 8 ml. The gross differences encountered would doubtless be evident in some degree under any conditions, but the details of the reaction in any individual case could not be expected to be always the same. Our results, although somewhat different from, are not therefore necessarily discordant with, the few previously recorded ones mentioned at the beginning of this paper [Rohde, 1918; Wada, 1930; Hunter, 1938]. Most of these, in any case, were obtained by the original volumetric technique of Van Slyke [1913].

As has been stated, our interest in the problem was purely empirical. We have made no study of the mechanism of the reaction between nitrous acid and the uramido group, or of the mode of operation of the factors which appear to modify its course.

Summary

Data are reported exemplifying the behaviour of various uramido-acids in the nitrous acid method for the determination of amino-nitrogen, and showing that this behaviour is far from uniform. Under certain specified conditions the amount of nitrogen evolved in 2·5 hr. varies from 0·66 to 2 atoms. The observed differences can, to a considerable extent, be related to structural differences in the various acids examined.

REFERENCES

Boon & Robson (1935). *Biochem. J.* **29**, 2573.
Boyd (1933). *Biochem. J.* **27**, 1838.
Dakin (1910). *Amer. chem. J.* **44**, 48.
—— (1915). *J. chem. Soc.* **107**, 434.
—— & Dudley (1914). *J. biol. Chem.* **17**, 29.
Dunn & Schmidt (1922). *J. biol. Chem.* **53**, 401.
Felix & Müller (1936). *Hoppe-Seyl. Z.* **240**, 1.
Gornall & Hunter (1939). *Biochem. J.* **33**, 170.
Hunter (1938). *Biochem. J.* **32**, 826.
Lippich (1908). *Ber. dtsch. chem. Ges.* **41**, 2953.
McMeekin, Cohn & Weare (1936). *J. Amer. chem. Soc.* **58**, 2173.
Rohde (1918). *J. biol. Chem.* **36**, 467.
Van Slyke (1913). *J. biol. Chem.* **16**, 121.
—— (1929). *J. biol. Chem.* **83**, 425.
Wada (1930). *Biochem. Z.* **224**, 420.
West (1918). *J. biol. Chem.* **34**, 187.

29. THE INFLUENCE OF GRADED DOSES OF VITAMIN A UPON PATHOLOGICAL CHANGES IN THE CENTRAL NERVOUS SYSTEM OF THE RAT, WITH SUGGESTIONS FOR A PROPHYLACTIC ASSAY OF THE VITAMIN

By J. T. IRVING and M. B. RICHARDS

From the Rowett Research Institute, Aberdeen

(*Received 30 December 1939*)

The quantitative determination of vitamin A is a problem which has formed the subject of much research. The unsatisfactory nature of the biological assay, when carried out by the usual curative method, has led to many attempts to find a reliable chemical or physical method for the determination. Good results seem to have been recently obtained with the spectrophotometric method, and Barthen & Leonard [1937] consider that this method can be adopted as an alternative for the biological test in determining the vitamin A content of fish liver oils. According to other authors, colour and vitameter tests are only supplementary to the biological test and cannot replace it [e.g. Black *et al.* 1938; Haines & Drummond, 1938; Møllgaard, 1938]. Hence the necessity for a reliable method of biological assay still exists.

In discussing results obtained by the curative method, reference is continually being made to the large margin of error inherent in the method. Richards & Simpson [1934] adduced much evidence regarding the unreliability of the method and showed that the differences in the pathological condition of the animals at the end of the depletion period are such that variations in response to vitamin A dosing are inevitable. A survey of the literature since that date indicates that little attention has yet been paid to this question of the pathological condition of the animals and to the impossibility of securing uniformity of condition at the time when the curative test begins. Freudenthal [1938] supports the view that the curative method is unreliable and advocates the preventive method, but most workers seem content to assume with Munsell [1938] that it is possible to obtain results with the growth method (i.e. curative method) "that have as high a degree of accuracy as can be expected of procedures in which animals are used as reagents". We are prepared to admit that the degree of accuracy may be as high as can be expected when the test animals are depleted of vitamin A before use; but it seems highly probable that much greater accuracy could be attained if a method were adopted which would ensure greater uniformity in the experimental material. A prophylactic test, starting from the time of weaning, on young rats in which the reserves of vitamin A have been kept low, seems to offer more prospect of success. It is objected that a prophylactic test would require much longer time to carry out, and this may be true where "increase in weight" is taken as the criterion in the assay of vitamin A. It would not be difficult to bring forward objections to the use of this criterion, and apparently other methods suggested, such as the "xerophthalmia" method and the "vaginal contents" method, do not possess advantages which outweigh the convenience of the "increase in weight" method [Coward, 1938]. The necessity for a new criterion seems therefore to be indicated.

Richards & Simpson [1934] suggested that a protective method should be adopted with the object of finding the dose of vitamin A necessary to keep the animal in healthy condition as evidenced by post-mortem examination. Subsequent work at this Institute makes it possible now to amplify this suggestion in a more practicable manner. Irving & Richards [1938] have shown that, in young rats on a vitamin A-free diet, degeneration occurs in the *funiculus praedorsalis* in the *medulla oblongata* with a constancy which is in marked contrast with the variability in pathological condition met with in other parts of the body. Since the degeneration is progressive and increases in severity with time from about 21 days onwards, it seemed possible that, by giving graded doses of vitamin A to the young animals and killing all members of a series at the same time, one might get a gradation in the severity of the lesion corresponding with the gradation of the dose.

Experimental

A preliminary experiment was carried out on the above lines, giving daily doses of 0, 1, 2, 4, 8 and 16 I.U. to six series of male litter-mates, which were fed on our usual vitamin A-free diet. In addition, two rats received doses of 3 I.U. and two received 6 I.U. A vitamin A concentrate in arachis oil, supplied by Glaxo Laboratories Ltd., was used for these tests. The rats belonging to five of the series were killed after 47 days on experiment, and those belonging to one series after 42 days, when the negative control animal was nearly moribund. The histological work was carried out by the method already described by Irving & Richards [1938]. The results for this preliminary experiment are given in Section 1 of Table 1, in which the degree of severity of the lesions is indicated by a number.

Table 1. *Degree of severity of lesions in the medulla of male rats after varying doses of vitamin A, all animals of a litter being killed on the same date*

The severity of the lesions is indicated by a number according to the following scheme:

Stage 1–: a very few fibres degenerated.
Stage 1 : a few scattered fibres degenerated.
Stage 2 : well-marked degeneration.
Stage 3 : very marked degeneration.
Stage 4 : heavy degeneration.

Section	Litter	Days on diet	Dose of vitamin A in International Units										
			0	⅓	⅔	1	1·5	2	3	4	6	8	16
1	A	47	4			2–3		1	1	1		1–	1–
	B	47	4			3		1		1–		?1–	1–
	C	47	4			1		1–		1		1–	1–
	D	47	4			2		1–	1–	1–	1–	1–	1–
	E	47	4			2		1–		1–	1–	1–	1–, 1–
	F	42	4			2		1		1–		1–	
2	C_1	50	3–4	4	3	2	1–						
	D_1	42	4	—	2–3	1–	1–	1–					
	F_1	52	—	3	3	3	1	1–		Food *ad lib.*			
	H	49	4	4	—	3	1	—	1				
	I	52	3–4	3	3	2	1	1					
3	E_1	52	4	4	4	2–3	1	1–	1	Food intake			
	G	49	3–4	4	2	2–3	1–	1–	1	equalized for litter			

It was found that the range of doses of vitamin A given in this experiment was unsuitable for showing the desired gradation in the lesions. Well-marked degeneration was present in all except one of the animals receiving 1 unit, but the

lesion was absent from all those which had received 2 or more units. (It is to be noted that the degree of degeneration represented by the numbers 1 and 1– is of no significance since the few scattered fibres thus denoted are found equally in positive controls and stock rats.)

The experiment was therefore repeated on similar lines but using smaller doses of vitamin A, viz. 0, 1/3, 2/3, 1, 1·5, 2 and, in a few cases, 3 units (Table 1, Section 2). In a further experiment two series of male rats had the food intake controlled so that all rats of one litter received the same amount of food; the results of these two series are shown in Section 3 of the table. The rats included in Sections 1 and 2 received food *ad lib.* The animals of each series were killed when the negative control was practically moribund.

It will be seen that in six of the seven series in Sections 2 and 3 there was a sharp demarcation between rats receiving 1 unit of vitamin A and those receiving 1·5 units. The lesion, which was still well marked in rats receiving the smaller dose, had disappeared from rats receiving 1·5 units. These animals were killed after periods of 49–52 days on experiment. In one series which was killed after 42 days, the demarcation occurred between the doses of 2/3 and 1 unit. It is possible that this difference may be due to the shorter period on experiment, but it is to be noted that of the litters in Section 1 killed at 47 days, the animal of litter C receiving 1 unit did not show a definite lesion in the medulla, while the corresponding animal of litter F, killed at 42 days, showed quite marked degeneration. The difference is therefore more probably due to individual variation. The control of the food intake, which was equalized for litters E and G (Section 3), apparently made no difference to the results, the demarcation for these two litters also occurring sharply between 1 and 1·5 units.

Discussion

A study of the data in Table 1 makes it clear that the severity of the lesion in the medulla in a prophylactic biological test is well adapted for evaluating the vitamin A content of a food substance. The constancy of the change at various dosage levels of vitamin A is in great contrast with the variability in weight response reported in animals examined by the curative method [Richards & Simpson, 1934; Coward, 1938 (p. 17)]. The working out of the exact method of procedure for comparing an unknown with a standard substance may be left to those to whom routine assay is a matter of importance. The details of our technique in regard to the breeding of the rats and the histological method adopted are to be found in our earlier paper [Irving & Richards, 1938], in which also are given our reasons for considering the Marchi technique reliable, if due precautions are taken. The procedure is simple and, although it may take 4–5 weeks before the final result is obtained, the stages before the cutting of the sections occupy little time and the whole process can be carried out as a simple working routine. After a little practice the maximum degree of degeneration present in any series of sections can be rapidly assessed.

It is possible that the time required might be reduced by using the short Marchi technique described by Stewart [1936]. We have not actually tried out this technique as we have found our own method very satisfactory and have felt no urgent need for hastening the results. We prefer to avoid formalin fixation in carrying out the Marchi method, but those to whom quick results are of importance might find the method of value.

Objection has been raised that the prophylactic test takes longer to perform than the curative method. It has to be remembered that in the method here suggested time and money are saved at the feeding stage of the experiment.

Most of the animals in our tests were killed after 47–52 days of feeding, but it is probable that for routine assay 42 or even 35 days would be preferable. Our previous work showed that after 35 days on the vitamin A-free diet all rats, both male and female, whose vitamin A intake had been restricted before weaning, showed stages 3 to 4 of the degeneration. If the rats receiving graded doses were killed at this stage, it seems likely that a better differentiation might be secured between the 0, 1/3 and 2/3 unit doses. When the animals are killed at a later stage, as in our tests, the degeneration tends to be equally severe for all these doses. In a curative assay the rats may well take 35 or more days for the preliminary period alone and they have then to be continued for a test period of 3–5 weeks. The total time taken for a prophylactic assay by this method need thus be little more than is required for an ordinary curative test.

It has to be remembered also that rats in a curative test frequently have to be rejected after the preliminary period because their weight renders them "unsuitable" for the test, and in the test period there may be losses by death which also reduce the number of available animals. In the results reported above no animals were rejected for any reason, and there would indeed be no need for any rejections in a test of this type.

It must not be assumed that we consider 1·5 units a sufficient dose, when given prophylactically from weaning, to meet the young rat's requirement of vitamin A. We have found from recent work on the teeth [Irving & Richards, 1939] that the rat's requirement for vitamin A seems to increase with age, since a dose which is sufficient to keep the teeth normal at 7 weeks proves inadequate if the animal is kept for over 17 weeks on the diet. It is thus impossible to state at present what is the amount of vitamin A required to bring an animal to maturity. The results quoted above indicate, however, the daily amount needed to avert the lesion of the medulla during the early period of rapid growth.

SUMMARY

1. Rats were placed at weaning on a vitamin A-free diet and given graded doses of vitamin A. They were killed after about 7 weeks and their medulla was examined by Marchi's method. Degeneration was found in the *funiculus praedorsalis* in those receiving 1 I.U. or less per day but not in those getting 1·5 I.U. or more, the demarcation being extremely sharp.

2. It is suggested that this procedure might be adapted to form the basis of a prophylactic assay of vitamin A. The main source of error in the curative method, in which the test is carried out on animals which may differ widely in pathological condition, is thereby avoided. Further, the procedure is simple and quick enough to be suitable for routine assay.

REFERENCES

Barthen & Leonard (1937). *J. Amer. pharm. Ass.* **26**, 515.
Black, Green, Sassaman & Sabo (1938). *J. Amer. pharm. Ass.* **27**, 199.
Coward (1938). The Biological Standardisation of the Vitamins. London: Baillière, Tindall & Cox.
Freudenthal (1938). *Ugeskr. Laeg.* **100**, 1346.
Haines & Drummond (1938). *Analyst*, **63**, 335.
Irving & Richards (1938). *J. Physiol.* **94**, 307.
—— —— (1939). *Nature, Lond.*, **144**, 908.
Mellgaard (1938). *Ugeskr. Laeg.* **100**, 1317.
Munsell (1938). *J. Amer. med. Ass.* **111**, 245.
Richards & Simpson (1934). *Biochem. J.* **28**, 1274.
Stewart (1936). *J. Path. Bact.* **43**, 339.

LINDA HALL LIBRARY
KANSAS CITY, MO.

30. STUDIES OF CALCIUM AND PHOSPHORUS METABOLISM IN RELATION TO THE CHEMICAL STRUCTURE OF BONE

1. EXPERIMENTS WITH LAYING BIRDS

By CYRIL TYLER

From the Royal Agricultural College, Cirencester

(*Received 6 November 1939*)

In a recent paper Common [1938] has suggested that the skeleton of a laying bird undergoes modifications in composition due to alterations in the ratio of carbonate CaO to total CaO and in the ratio of residual CaO to total CaO. He defines residual CaO as that CaO not present in the form of $Ca_3(PO_4)_2$. Brook *et al.* [1934] have stated that it is possible to change the ratio of $Ca_3(PO_4)_2$ to $CaCO_3$ in the bones of rats by dietetic means, whilst Burns & Henderson [1936] have shown that, within limits, the ratio of carbonate to total Ca in bone appears to be a measure of its age. Further, Marek *et al.* [1937] report that feeding $CaCO_3$ to young pigs increased the Ca : P ratio of the bones.

Thus there appears to be some evidence that the composition of bone can alter, which in turn suggests that it is possible for bone to gain or lose Ca without gaining or losing an exactly equivalent amount of P. If this be admitted, then the possibility arises that Ca may be gained or lost from the bone without any change in its P content and *vice versa*. Such conceptions are contrary to the generally accepted idea that the composition of the skeleton is fairly constant and contrary to the evidence of many experiments which indicate concomitant losses or gains of Ca and P. It was therefore decided to attempt to obtain further information on this subject.

Experiments carried out by the author [Tyler, 1935] with a view to comparing the relative efficiencies of various Ca compounds in egg production yielded results which on re-examination furnish some interesting data concerning changes in the chemical composition of the bone during egg laying.

In the experiments referred to, two Rhode Island Red pullets (or hens) were housed in separate metabolism cages and fed on weighed amounts of a basal ration of commonly used poultry foods, which was low in Ca but adequate in every other respect. Distilled water was constantly available for drinking purposes and the basal ration was supplemented in the first experiment with calcium carbonate, in the second with calcium sulphate and in the third with calcium gluconate, the supplement providing equal quantities of Ca in each case. At the same hour of each day food residues, faeces and eggs were collected from each bird separately and fresh food and water were given. Food, food residues, faeces, egg shell and egg content were analysed for Ca and P and daily balances were struck. Each experiment lasted 35 days and was preceded by a preliminary period of about 5 days on the same diet. Different pairs of birds were used for each experiment and the three experiments were not carried out concurrently.

In the 1st and 3rd experiments one of the two birds moulted so that the results are only available for four laying birds. It is hoped to consider the results for the two moulting birds in a later paper.

Methods of analysis

Ca was estimated by precipitating it as oxalate and titrating with standard potassium permanganate. P was estimated by precipitating it as ammonium phosphomolybdate and titrating with standard alkali, using the method of Pemberton & Neumann as modified by Richards & Godden [1924].

Results

For ease of reference the birds have been designated C2, S1, S2 and G2, indicating respectively the bird receiving calcium carbonate, the two birds receiving calcium sulphate and the bird receiving calcium gluconate.

Table 1 gives the relevant data regarding these four birds.

Table 1

Bird	No. of eggs laid	% Ca in food	% P in food	Ca : P of food	Ca balance over period g.	P balance over period g.	Ca : P of balance
C2	18	1·68	0·40	4·20	−3·11*	−1·57*	1·98
S1	17	1·76	0·37	4·76	−5·83	−0·49	11·90
		1·76	0·43	4·09			
S2	21	1·76	0·37	4·76	−8·11†	−0·67†	12·10
		1·76	0·43	4·09			
G2	18	1·65	0·32	5·16	+4·55*	−1·60*	—

Note. The two sets of Ca and P values for the food given in the case of birds S1 and S2 indicate that these birds received the same ration but during the last 14 days of the experimental period the P content was slightly higher than during the first 21 days. This was due to the use of a fresh consignment of feeding stuffs after the first 21 days and not to any radical change in the nature of the ration.

* 34 day balance
† 32 day balance
} due to breakage of eggs on other days.

If the values for the balances of Ca and P shown in Table 1 represent the changes in bone Ca and P then obviously there must be some alteration in the composition of the bones since the Ca/P ratio of bone is 2·2 : 1 and in only one case, namely C2, is the Ca/P ratio of the balance anywhere near 2·2 : 1; and further, in the case of G2 a positive Ca balance is associated with a negative P balance.

It is felt that as long as the birds are neither gaining nor losing flesh rapidly (and these birds were fairly constant in weight throughout the period) then the total Ca and P content of the tissues other than bone will remain at least sufficiently constant so as not to affect calculations regarding the relatively large quantities of Ca and P taking part in bone changes during a laying period. It is only when tissue other than bone is being rapidly broken down or built up that such calculations are likely to be seriously upset.

In further support of this contention are the values obtained if it is alternatively assumed that bone breaks down or builds up on the basis of a Ca : P ratio of 2·2 : 1. In such a case the values obtained for Ca or P stored in or lost from tissues other than bone are far in excess of changes which the tissues could stand. In the case of bird G2, for example, if the Ca : P ratio of bone changes is 2·2 : 1 then:

(*a*) assuming that no Ca changes take place in the tissues, the balance of Ca will represent the bone changes of Ca and from this a simple calculation shows that the P lost from the tissues would be 3·67 g.

or (*b*) assuming that no P changes take place in the tissues, the balance of P will represent the bone changes of P, and from this a simple calculation shows that the Ca gained by the tissues would be 8·07 g.

Such values represent the two extremes of change but calculation of intermediate values reveals changes equally outside the range of physiological probability.

Similarly improbable figures can be obtained from the data for birds S 1 and S 2.

Thus the assumption appears justified that the values for the Ca and P balances in these cases represent, in the main, changes in the bone Ca and P, and that the bones of these birds must therefore be changing in composition.

It was felt that little further information could be obtained from total balances over the whole experiment and that some evidence was needed as to what part of the bone compound was changing. With this in mind the following calculation, similar to that used by Common [1938], was made for each day of each experiment.

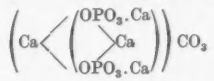
The P balance figure was multiplied by the factor 1·93 (Ca : P ratio of $Ca_3(PO_4)_2 = 1{\cdot}93 : 1$) to give the value of the Ca lost or gained by the $Ca_3(PO_4)_2$ portion of the bone. Then, by subtracting this figure from that for the total Ca

Table 2. *Bird C 2. Ca_P and Ca_R lost or gained by bone, the retention of Ca (Ca_A) and egg shell Ca (Ca_S), (g.)*

	Laying days				Non-laying days		
Day	Ca_P	Ca_R	Ca_A	Ca_S	Ca_P	Ca_R	Ca_A
1	—	—	—	—	−0·39	+1·55	+1·16
2	−0·95	−0·29	+0·83	2·03	—	—	—
3	−0·69	+0·09	+1·41	1·98	—	—	—
4	—	—	—	—	+0·46	+0·75	+1·21
5	−0·23	−0·62	+1·01	1·82	—	—	—
6	Omitted because of incomplete data						
7	—	—	—	—	−0·81	+2·01	+1·20
8	+0·37	−1·78	+0·68	2·04	—	—	—
9	+0·04	−0·68	+1·27	1·85	—	—	—
10	—	—	—	—	−1·20	+2·34	+1·14
11	+0·33	−1·41	+1·00	2·04	—	—	—
12	−0·35	−0·87	+0·70	1·88	—	—	—
13	—	—	—	—	−0·31	+1·58	+1·27
14	+0·31	−1·23	+1·16	2·03	—	—	—
15	−0·41	−0·40	+1·22	1·99	—	—	—
16	—	—	—	—	+0·69	+0·43	+1·12
17	−0·66	−1·27	+0·00	1·87	—	—	—
18	—	—	—	—	+0·44	−0·01	+0·43
19	—	—	—	—	+0·60	+0·17	+0·77
20	—	—	—	—	+0·41	+0·24	+0·65
21	−0·62	−0·53	+0·74	1·84	—	—	—
22	—	—	—	—	+0·58	−0·02	+0·56
23	—	—	—	—	+0·58	+0·22	+0·80
24	—	—	—	—	+0·37	+0·39	+0·76
25	−0·60	−0·72	+0·68	1·94	—	—	—
26	—	—	—	—	+0·48	+0·36	+0·84
27	—	—	—	—	−0·31	+1·26	+0·95
28	+0·35	−1·32	+1·00	1·91	—	—	—
29	−0·75	−0·29	+1·04	2·03	—	—	—
30	—	—	—	—	+0·44	+0·24	+0·68
31	−0·41	−0·58	+1·10	2·04	—	—	—
32	—	—	—	—	−0·50	+1·42	+0·92
33	+0·21	−1·32	+1·03	2·08	—	—	—
34	—	—	—	—	−0·41	+1·27	+0·86
35	−0·10	−1·04	+0·89	1·99	—	—	—
Sum	−4·16	−14·26	+15·76	33·35	+1·12	+14·20	+15·32
Mean	−0·24	−0·84	+0·93	1·96	+0·07	+0·84	+0·90

balance the value of the residual Ca lost or gained by the bones was obtained. Tables 2, 3, 4 and 5 show the values obtained by this calculation and in addition the amounts of Ca in the egg shells and the amounts of Ca retained on any one day. This latter figure is not a measure of actual absorption but is simply the difference between the intake of food Ca and the output of "faecal" Ca. It must also be remembered that "faecal" Ca or any other constituent of the "faeces" represents, in the case of birds, both urinary and faecal excretion.

There has been, and still is, some controversy about the chemical structure of bone. Various formulae have been advanced such as $CaCO_3 . nCa_3(PO_4)_2$ where $n=2$ [Bogert & Hastings, 1931] $Ca((Ca_3(PO_4)_2)_6)(OH)_2$ [Morgulis, 1931;



Gassmann, 1931]. But if, when discussing the chemical composition of bone, the terms "Ca of $Ca_3(PO_4)_2$" and "residual Ca" are used the controversy is avoided and has no influence upon the results. These two terms can with convenience be abbreviated to Ca_P and Ca_R respectively and such abbreviations will be used in this paper.

Table 3. *Bird S1. Ca_P and Ca_R lost or gained by bone, the retention of Ca (Ca_A) and egg shell Ca (Ca_S), (g.)*

	Laying days				Non-laying days		
Day	Ca_P	Ca_R	Ca_A	Ca_S	Ca_P	Ca_R	Ca_A
1	—	—	—	—	+0·17	+0·30	+0·47
2	−0·54	−0·41	+0·57	1·46	—	—	—
3	—	—	—	—	+0·39	+0·25	+0·64
4	—	—	—	—	+0·42	+0·05	+0·47
5	—	—	—	—	+0·29	−0·08	+0·21
6	—	—	—	—	+0·10	+0·45	+0·55
7	—	—	—	—	+0·41	−0·07	+0·34
8	−0·66	−0·45	+0·62	1·68	—	—	—
9	—	—	—	—	+0·29	+0·48	+0·77
10	−1·00	−0·71	+0·15	1·80	—	—	—
11	—	—	—	—	+0·31	+0·18	+0·49
12	—	—	—	—	+0·41	+0·25	+0·66
13	−0·79	−0·81	+0·31	1·86	—	—	—
14	—	—	—	—	−0·04	+0·97	+0·93
15	−0·17	−1·30	+0·44	1·87	—	—	—
16	−0·64	−0·48	+0·80	1·88	—	—	—
17	—	—	—	—	−0·52	+1·18	+0·66
18	+0·27	−1·20	+0·79	1·66	—	—	—
19	−0·58	−0·36	+0·85	1·74	—	—	—
20	—	—	—	—	−0·10	+1·16	+1·06
21	−0·19	−0·67	+0·79	1·59	—	—	—
22	+0·14	−1·03	+0·66	1·49	—	—	—
23	−0·37	−0·69	+0·54	1·55	—	—	—
24	—	—	—	—	+0·02	+0·75	+0·77
25	+0·39	−0·92	+0·81	1·29	—	—	—
26	−0·21	−0·40	+0·93	1·48	—	—	—
27	—	—	—	—	−0·19	+1·21	+1·02
28	+0·25	−0·87	+0·88	1·45	—	—	—
29	—	—	—	—	+0·56	−0·21	+0·35
30	—	—	—	—	+0·10	+0·70	+0·80
31	+0·15	−1·14	+0·57	1·51	—	—	—
32	−0·17	−0·68	+0·72	1·52	—	—	—
33	—	—	—	—	−0·27	+0·90	+0·63
34	+0·39	−1·09	+0·74	1·39	—	—	—
35	—	—	—	—	+0·50	−0·21	+0·29
Sum	−3·73	−13·21	+11·17	27·19	+2·85	+8·24	+11·11
Mean	−0·22	−0·78	+0·66	1·60	+0·16	+0·46	+0·62

Table 4. *Bird S2. Ca_P and Ca_R lost or gained by bone, the retention of Ca (Ca_A) and egg shell Ca (Ca_S), (g.)*

	Laying days				Non-laying days		
Day	Ca_P	Ca_R	Ca_A	Ca_S	Ca_P	Ca_R	Ca_A
1	—	—	—	—	+0·27	+0·26	+0·53
2	−0·66	−0·55	+0·56	1·69	—	—	—
3	—	—	—	—	−0·44	+1·12	+0·68
4	+0·27	−1·33	+0·77	1·77	—	—	—
5	−0·81	−0·63	+0·50	1·88	—	—	—
6	—	—	—	—	−0·48	+1·29	+0·81
7	+0·27	−1·59	+0·61	1·86	—	—	—
8	−0·66	−0·75	+0·57	1·90	—	—	—
9	—	—	—	—	−0·10	+1·14	+1·04
10	+0·21	−1·13	+0·87	1·72	—	—	—
11	−0·60	−0·58	+0·68	1·78	—	—	—
12	—	—	—	—	+0·35	+0·61	+0·96
13	Omitted because of incomplete data						
14	—	—	—	—	+0·25	−0·67	−0·42
15	−0·35	−0·69	+0·79	1·77	—	—	—
16	—	—	—	—	−0·25	+1·25	+1·00
17	−0·19	−0·55	+0·87	1·55	—	—	—
18	+0·23	−1·19	+0·59	1·49	—	—	—
19	—	—	—	—	−0·23	+1·23	+1·00
20	+0·15	−0·89	+0·88	1·56	—	—	—
21	−0·54	−0·19	+0·89	1·55	—	—	—
22	—	—	—	—	−0·10	+0·90	+0·80
23	Omitted because of incomplete data						
24	Omitted because of incomplete data						
25	—	—	—	—	+0·66	−0·07	+0·59
26	+0·19	−1·82	+0·78	2·31*	—	—	—
27	—	—	—	—	+0·10	+0·78	+0·88
28	+0·35	−0·99	+0·98	1·56	—	—	—
29	−0·64	−0·70	+0·66	1·89*	—	—	—
30	—	—	—	—	+0·64	+0·28	+0·92
31	+0·35	−1·02	+0·99	1·60	—	—	—
32	—	—	—	—	−0·10	+1·08	+0·98
33	+0·25	−0·83	+1·05	1·56	—	—	—
34	−0·52	−0·77	+0·69	1·87*	—	—	—
35	—	—	—	—	+0·83	+0·21	+1·04
Sum	−2·70	−16·20	+13·73	31·34	+1·40	+9·41	+10·81
Mean	−0·15	−0·90	+0·76	1·74	+0·10	+0·67	+0·77

* Indicates double-yolked egg.

Turning now to Tables 2–5 it will be seen that Ca_P shows no definite trend either on days when eggs are laid or on days when no eggs are laid; sometimes the values indicate a loss from the bone and sometimes a gain. The result is that on egg-laying days the daily mean value for Ca_P is, in the case of all four birds, a very small value on the negative side; on the other hand, on days when no eggs are laid the daily mean value for Ca_P shows a small gain in three birds, and in the other, G2, a small loss. The total Ca_P changes for the whole experiment represent a loss in each case.

It would thus appear that the Ca_P in all these four laying hens fluctuates from day to day, that the direction of these fluctuations is not specifically determined by the laying of an egg or by the non-laying of an egg, and that the general trend is towards a loss of Ca_P over the whole period.

A different state of affairs exists however in the case of Ca_R. With only a few exceptions there is quite a large loss of Ca_R on laying days and quite a large gain of Ca_R on non-laying days. Daily mean values for the different birds show gains on non-laying days ranging from 0·46 to 1·20 g. and losses on laying days ranging

Table 5. *Bird G2. Ca_P and Ca_R lost or gained by bone, the retention of Ca (Ca_A) and egg shell Ca (Ca_S), (g.)*

	Laying days				Non-laying days		
Day	Ca_P	Ca_R	Ca_A	Ca_S	Ca_P	Ca_R	Ca_A
1	+0·25	−1·09	+1·18	1·96	—	—	—
2	−0·39	+0·33	+1·77	1·79	—	—	—
3					Omitted because of incomplete data		
4	−0·02	−0·73	+1·20	1·90	—	—	—
5	−0·52	+0·05	+1·40	1·81	—	—	—
6	—	—	—	—	+0·46	+0·73	+1·19
7	—	—	—	—	−0·21	+1·37	+1·16
8	0·00	−0·70	+1·30	1·94	—	—	—
9	−0·48	−0·14	+1·28	1·84	—	—	—
10	—	—	—	—	+0·21	+0·66	+0·87
11	−0·42	+0·19	+1·46	1·64	—	—	—
12	—	—	—	—	+0·37	+0·54	+0·91
13	—	—	—	—	−0·46	+1·68	+1·22
14	+0·19	−1·03	+1·07	1·85	—	—	—
15	—	—	—	—	−0·06	+1·70	+1·64
16	+0·02	−1·05	+0·97	1·93	—	—	—
17	—	—	—	—	−0·42	+1·53	+1·11
18	+0·08	−1·24	+0·77	1·89	—	—	—
19	—	—	—	—	−0·31	+1·42	+1·11
20	+0·17	−0·93	+1·24	1·94	—	—	—
21	—	—	—	—	+0·08	+1·04	+1·12
22	+0·33	−0·45	+0·94	0·99	—	—	—
23	—	—	—	—	−0·54	+1·54	+1·00
24	+0·02	−0·90	+1·09	1·91	—	—	—
25	−0·73	+0·19	+1·50	1·98	—	—	—
26	—	—	—	—	+0·39	+0·38	+0·77
27	—	—	—	—	−0·33	+1·47	+1·14
28	+0·04	−0·92	+0·93	1·76	—	—	—
29	—	—	—	—	−0·52	+1·70	+1·18
30	+0·15	−1·01	+1·10	1·91	—	—	—
31	—	—	—	—	−0·35	+1·45	+1·10
32	0·00	−0·93	+0·90	1·77	—	—	—
33	—	—	—	—	+0·37	+0·37	+0·74
34	—	—	—	—	−0·54	+1·54	+1·00
35	+0·06	−1·13	+0·89	1·90	—	—	—
Sum	−1·25	−11·49	+20·99	32·68	−1·86	+19·12	+17·26
Mean	−0·07	−0·64	+1·17	1·82	−0·12	+1·20	+1·08

from 0·64 to 0·90 g. The total Ca_R changes, however, reveal radical differences in each bird. C2 finished the experiment showing neither a gain nor a loss, S1 and S2 both showed losses and G2 showed a gain.

These findings strongly suggest that it is the Ca_R portion of the bone which undergoes relatively large fluctuations during a laying period and further that the sum total of these fluctuations is considerably affected by the nature of the Ca compound fed to the birds.

The final point to be noted from these Tables is that the daily mean value for the Ca retained (from now on referred to as Ca_A) is almost identical on laying and non-laying days for each individual bird but that there are large variations between the four birds, indicating once more a probable relationship to the kind of Ca compound fed. This naturally leads to a consideration of the relationships between Ca_P, Ca_R and Ca_A.

The daily mean values for each bird for laying days have been collected in Table 6 along with the daily mean values for the Ca of the shell and of the egg contents.

Table 6. *Daily mean values of Ca_P, Ca_R and Ca_A for laying days and the daily mean values for egg shell and egg contents Ca (g.)*

Bird	Ca_P available for egg	Ca_R available for egg	Ca_P + Ca_R	Ca_A available for egg	Ca in shell	Ca in content
C 2	0·24	0·84	1·08	0·93	1·96	0·05
S 1	0·22	0·78	1·00	0·66	1·60	0·06
S 2	0·15	0·90	1·05	0·76	1·74	0·07
G 2	0·07	0·64	0·71	1·17	1·82	0·05

Ca_P available for egg is the negative value of Ca_P given in Tables 2–5. This applies also to the values Ca_R and $Ca_P + Ca_R$.

From Table 6 it would appear that on the average, whatever the Ca compound fed, there is a maximum amount of Ca available from the bones ($Ca_P + Ca_R$) which is in the region of 1 g.; this, plus the amount available from the food (Ca_A), decides the amount of Ca in the shell. The small amount of Ca in the egg contents can be neglected here. Thus, provided that the maximum amount of Ca comes from the bones, the final amount of Ca in the shell will be decided by the quantity of Ca retained from the food. This finding lends support to similar ideas expressed by Tyler [1935]. The bird G 2 stands alone; in her case only 0·71 g. Ca came from the bones and 1·17 g. from the food. This lowered contribution from the skeleton suggests that in this case the skeleton and the food together could supply more than was required by the shell and therefore a saving was effected on the quantity of Ca withdrawn from the skeleton. The author, using daily averages for a number of birds, has however shown that the maximum amount of Ca which a bird can retain from her food is in the region of 1 g.; therefore the saving effected by food Ca on skeletal reserves is limited.

Briefly then the maximum Ca content of a shell (for Rhode Island Reds) is somewhere about 2 g. and the skeleton will supply about 1 g. and the food about 1 g. If the supply from the food is less than 1 g. then the shell will decrease in Ca content, whilst if the maximum shell value for any individual bird is less than 2 g. and the food supplies 1 g. then there will be an automatic saving of bone Ca.

It must be stressed here however that such statements are generalizations based on average values and when daily values are examined a more intimate relationship between Ca coming from the food (Ca_A) and that coming from the bones ($Ca_P + Ca_R$) is noted.

This is clearly seen when the daily values for ($Ca_P + Ca_R$) are plotted against Ca_A (Fig. 1). There is an obvious reciprocal relationship between the Ca provided by the food and that provided by the skeleton for the purpose of egg shell formation. This relationship holds over the range of values found in this experiment but whether it stretches beyond these ranges is a matter for conjecture. At this stage there is not sufficient evidence to warrant a discussion on the food and bone Ca supplies to the shell, i.e. as to how they interact and what other factors are effective.

The daily mean values of Ca_P, Ca_R and Ca_A for each bird on non-laying days have been collected in Table 7.

In Table 7 the sum of Ca_P and Ca_R equals Ca_A. Once more the maximum retention is about 1 g. Ca but with the bird G 2 the effort to keep within the range appears to have been made at the expense of a Ca_P loss. In other words, if the value of Ca_A is below 1 g. then part goes to Ca_P and part to Ca_R, but if the value of Ca_R exceeds 1 g. then there is a loss of Ca_P to keep the value of Ca_A within its limit. This is clearly seen in Fig. 2.

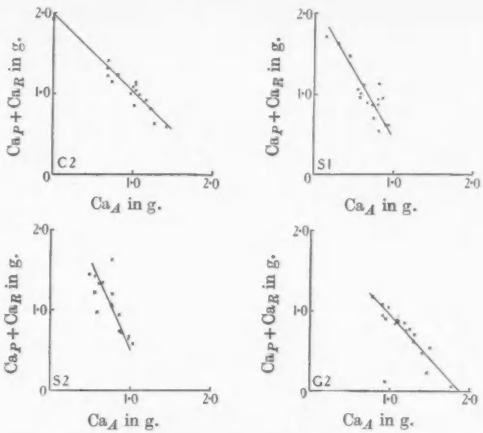


Fig. 1. Relationship between bone calcium ($Ca_P + Ca_R$) and food calcium (Ca_A) for egg shell formation. Four birds C 2, S 1, S 2 and G 2.

Table 7. *Daily mean values of Ca_P, Ca_R and Ca_A for non-laying days (g.)*

Bird	Ca_P to bone	Ca_R to bone	Ca_A to bone
C 2	0·07	0·84	0·90
S 1	0·16	0·46	0·62
S 2	0·10	0·67	0·77
G 2	−0·12	1·20	1·08

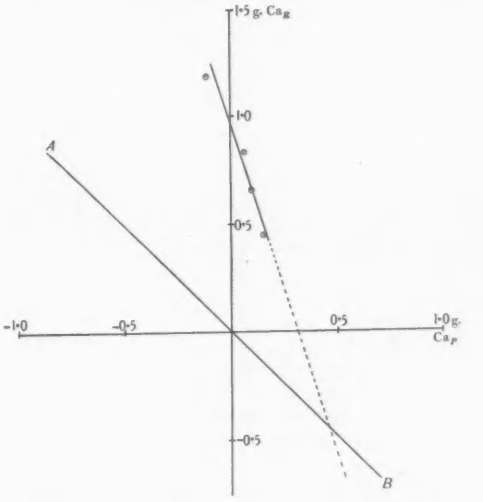


Fig. 2. Relationship of Ca_P to Ca_R gained or lost by the bone. Each point represents the daily mean value for one of the four birds on non-laying days. *AB* represents the calcium equilibrium line. To the N.E. is a total calcium gain; to the S.W. is a total calcium loss.

Further consideration of Fig. 2 reveals that, whilst in the N.E. quadrant of the graph there is depicted a gain of both Ca_P and Ca_R, in the N.W. quadrant a loss of Ca_P and a gain of Ca_R are represented. Theoretically if the line be continued downwards into the S.E. quadrant there exists the possibility of a gain of Ca_P and a loss of Ca_R, and a slight shifting of the line to the left would lead to a state of affairs in which there would be a loss of both Ca_P and Ca_R. From these very general considerations based on average values there appears to be a reciprocal relationship between Ca_P and Ca_R which relationship can provide theoretically for a loss of P with a gain of Ca, a loss of Ca with a gain of P or a loss or gain of both together. Such a conception is far more elastic and would provide a far more useful mechanism to a bird or animal than the usually accepted idea of a mechanism involving coincident losses or gains of Ca and P. In addition this suggested conception reconciles the two groups of conflicting evidence mentioned earlier in this paper.

It was decided to test the reciprocity of Ca_P and Ca_R more stringently by calculating the correlation coefficients of these values for each of the four birds and for laying and non-laying days. The reason for calculating these values for laying and non-laying days instead of for the whole experiment may be stated briefly. If there is the same reciprocal relationship both on the laying and the non-laying days then this relationship would still be revealed by a high correlation coefficient even when the figures for the whole experiment were taken. On the other hand, if the reciprocal relationship on laying days is different from that on non-laying days then a consideration of the experiment as a whole might give a very low correlation coefficient of no significance. Hence a consideration of correlation coefficients for both laying and non-laying days has no disadvantages but perhaps will reveal something which would otherwise be obscured.

The values of the various correlation coefficients are given in Table 8. All of them are negative and all are highly significant.

Table 8. *Correlation between Ca_P and Ca_R on laying and non-laying days for each bird*

Correlation coefficients (r) and the number of pairs of readings used in their calculation (n).

	Laying		Non-laying	
Bird	r	n	r	n
C2	−0·7883	17	−0·9606	17
S1	−0·6431	17	−0·9149	18
S2	−0·7114	18	−0·7702	14
G2	−0·8403	18	−0·9164	16

Such evidence greatly strengthens the suggested conception. To put this reciprocal relationship into graphical form the regression of Ca_R on Ca_P was calculated and the regression line drawn, and since the correlations are highly significant this line will lie fairly close to the regression line for Ca_P on Ca_R and hence is fairly representative of the true equation relating Ca_P and Ca_R.

These regression lines are shown in Fig. 3 and are drawn within the limits to which they refer, taking as limits the extreme values of Ca_R.

Consideration of Fig. 3 shows that the theoretically possible cases previously outlined are found in practice, namely, coincident gains or losses of Ca and P from the bone or Ca losses with P gains or Ca gains with P losses.

The fact that the regression lines fall into two distinct groups, one for laying and one for non-laying days, suggests that although the reciprocal relationships

exist for Ca_P and Ca_R on both laying and non-laying days yet these relationships are not in the same plane. Since it has been noted already that Ca_P fluctuates but little about zero on both laying and non-laying days the standard of $Ca_P = 0$ suggests itself as a basis for comparing the difference in the two reciprocal relationships. On this basis the range of gain of Ca_R on non-laying days is 0·70–1·05 g. and the range of loss of Ca_R on laying days is 0·75–1·05 g.

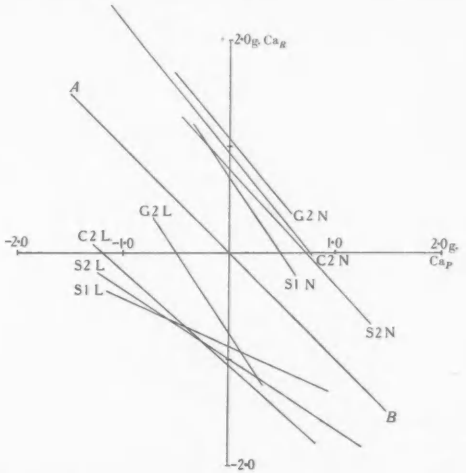


Fig. 3. Regression lines of Ca_R on Ca_P for the four birds. L = laying days; N = non-laying days. *AB* represents the calcium equilibrium line. To the N.E. is a total calcium gain; to the S.W. is a total calcium loss.

Therefore the position is this. For non-laying days there is, when $Ca_P = 0$, a gain of Ca_R of between 0·70 g. and 1·05 g. depending upon the bird and also upon the supply of Ca. If Ca_P shows a loss then Ca_R shows a larger gain; when Ca_P shows a gain, however, the value of the gain of Ca_R falls away and may even become a loss. On laying days there is, when $Ca_P = 0$, a loss of Ca_R of between 0·75 g. and 1·05 g. depending upon the Ca supply and upon the Ca content of the egg shell. If Ca_P shows a gain then Ca_R shows a larger loss, but when Ca_P shows a loss the value of the Ca_R loss falls and may even become a gain.

Discussion

It must first of all be clearly understood that any findings set forth here or any conclusions drawn refer only to the four birds under consideration and to the specific conditions of the experiments.

From the results already discussed there would appear to be a mechanism whereby the laying hen is prevented from losing too much Ca from her skeleton on laying days and from gaining too much on non-laying days. If averages over a whole experiment (35 days) are taken this mechanism seems to work in such a way that the laying hen can prevent the withdrawal of more than about 1·0 g. total Ca from her bones on any one day when an egg is laid and on days when no egg is laid no more than about 1·0 g. total Ca can be laid down in the bones. This

mechanism consists of a reciprocal relationship between Ca_P and Ca_R which acts in such a way that within limits there is a loss or gain of both Ca_P and Ca_R, but as soon as this loss or gain tends to pass beyond these limits in the case of one it is counteracted by an opposite movement of the other.

Much work is required before the mechanism of such changes can be elucidated, but working on the basis that much larger changes occur in the Ca_R than in the Ca_P it might be tentatively suggested that if amounts of Ca_R above a certain maximum are being mobilized then part of that Ca_R will be precipitated as Ca_P leading to a loss of Ca_R and a gain of Ca_P. On the other hand, if amounts of Ca_R above a certain maximum are being laid down, part of this Ca_R must come from a reversion of Ca_P to Ca_R with loss of P, the final result being a gain of Ca_R and a loss of Ca_P.

Whether the Ca_R is made up of $CaCO_3$, $Ca(OH)_2$ or a mixture of both, the large fluctuations of this portion of bone Ca would, in addition to Ca, only liberate or absorb ions which are easily removed or supplied by the body, namely, $(OH)'$ or $(CO_3)''$. The thesis that $(CO)_3''$ is the more likely ion for the purpose is supported by that school which believes bone to consist of $Ca_3(PO_4)_2$ and $CaCO_3$ and particularly by Klement [1936] who suggests that bone is made up of a hydroxyapatite lattice in which $CaCO_3$ crystals are deposited.

The final picture one might suggest is therefore a "real" skeleton of $Ca_3(PO_4)_2$ which remains fairly constant in amount whilst fluctuating depositions and withdrawals of Ca necessitated during an egg-laying cycle take the form of $CaCO_3$ whose breakdown or synthesis will not greatly upset the delicate ionic equilibrium. At the same time a limit is set to the total Ca changes by a reciprocal action between the $Ca_3(PO_4)_2$ of the "real" skeleton and the easily mobilizable $CaCO_3$.

Summary

1. An investigation into the Ca and P metabolism of poultry is described.

2. From the results of these experiments a value for the Ca of the $Ca_3(PO_4)_2$ of bone and for the residual Ca of bone gained or lost each day is calculated.

3. It is shown that the residual Ca of the bone is chiefly concerned in egg laying.

4. The relationship of food and bone Ca to egg shell Ca is mentioned.

5. By means of correlation coefficients it is shown that there is a reciprocal relationship between the Ca of calcium phosphate and the residual Ca of bone; the implications of this relationship are discussed.

The author is indebted to the University of Leeds under whose auspices the experimental work was carried out and particularly to Mr J. S. Willcox for his never failing interest. Also to Mr K. L. Robinson for his useful criticism of the ideas embodied in the paper.

REFERENCES

Bogert & Hastings (1931). *J. biol. Chem.* **94**, 473.
Brook, Smith & Smith (1934). *J. biol. Chem.* **104**, 141.
Burns & Henderson (1936). *Biochem. J.* **30**, 1207.
Common (1938). *J. agric. Sci.* **28**, 347.
Gassmann (1931). *Hoppe-Seyl. Z.* **201**, 284.
Klement (1936). *Ber. dtsch. chem. Ges.* **69**, 2232.
Marek, Wellmann & Urbanyi (1937). *Arch. wiss. prakt. Tierheilk.* **72**, 1.
Morgulis (1931). *J. biol. Chem.* **93**, 455.
Richards & Godden (1924). *Analyst*, **49**, 565.
Tyler (1935). Ph.D. Thesis, Univ. Leeds.

31. ANTI-α-GLUCOSIDASE[1]

BY SHLOMO HESTRIN

Department of Hygiene and Bacteriology, Hebrew University, Jerusalem

(*Received 7 December 1939*)

ABSENCE of dependable criteria renders differentiation between enzymes in crude biological preparations a task of much difficulty. Existing systems are generally based on differences in such properties as substrate specificity, *p*H function, activability or inactivability. Unfortunately, none of these properties can be shown in advance to be necessarily related exclusively to the enzymes themselves. On the contrary, it is becoming increasingly apparent that for some kinds of enzyme these properties are variable, being dependent on the nature of the accompanying substances present in the crude enzyme preparations employed [Oppenheimer, 1936]. It is therefore obvious that the task of differentiation is especially complicated when enzymes of overlapping or even identical substrate specificity have to be examined.

Among the oligases no final general decision as to the ultimate identity or non-identity of maltases and sucrases from different sources has been reached. Two different sucrases have been distinguished by Kuhn & Munch [1927] partly on the basis of differential behaviour to modified sucrose molecules. An analogous weapon has been applied by Leibowitz [1925, also Leibowitz & Mechlinski, 1926; Leibowitz & Hestrin, 1938; 1939] to the differentiation of the maltases. According to Leibowitz, mould and malt maltases are essentially different from yeast maltase, not only because of their very different physical properties but because the substrate specificity of the former, unlike that of the latter, does not extend to maltose derivatives which have suffered modification in the non-glucosidic residue. Yeast maltase which splits different α-glucosides as well as maltose is therefore termed glucosido-maltase (=α-glucosidase), malt and mould maltases which split only maltose [cf. Hestrin, 1939] are described as gluco-maltase.

In a series of papers, Weidenhagen [1933; 1934] has developed a theory of carbohydrase specificity which is incompatible with the existence of a glucosucrase or of a gluco-maltase distinct from yeast glucosido-maltase. According to Weidenhagen, carbohydrase specificity is determined in a qualitative sense exclusively by the ring structure, configuration, and C^1-stereoisomerism of the glycosidic residue of the substrate molecule, and is not qualitatively dependent on the non-glycosidic residue. It is therefore postulated by Weidenhagen that all maltases are identical with yeast maltase, which is termed α-glucosidase, and it is predicted that all maltases will hydrolyse different α-glucosides—including alkyl and aryl glucosides, sucrose and melezitose—as well as maltose. The discrepancies between this theory and an imposing list of experimental findings have been emphasized by different authors [e.g. Iwanoff *et al.* 1930; Karstrom, 1930; Tauber & Kleiner, 1933; Pringsheim *et al.* 1931; Myrback, 1931; Grassman *et al.* 1933; Hofmann, 1934; Leibowitz & Hestrin, 1939; Hestrin, 1939]. Nevertheless, the comparative simplicity of Weidenhagen's views continues to receive sympathetic comment [Oppenheimer, 1936].

[1] Cf. preliminary note by Leibowitz & Hestrin [1938]. The author is indebted to Dr J. Leibowitz for kind guidance throughout the present investigation.

The identification of yeast maltase with mould maltase, can only be explained on Weidenhagen's theory if it is shown that the differences found between these two maltase preparations are the result of differences in accompanying substances rather than of differences in the maltase molecules themselves. If Weidenhagen's system is to be retained, it must either be shown that yeast maltase is active to methyl α-glucoside because its substrate range has been extended by the presence of specific complement or activator, or it must be shown that mould maltase is inert to methyl α-glucoside because its specificity has been narrowed by the contracting effect of a substrate-specific inhibitor.

Various situations may arise in a mixture of yeast and mould maltases:

1. The yeast and mould maltases may be intrinsically different and their preparations devoid of secondary substances which differentially influence their respective substrate specificity and activity. Then the activity of a mould-yeast mixture to methyl α-glucoside per unit of yeast present will be equal to, but the unit activity to maltose will be greater than, the activity of the yeast component alone.

2. The yeast and mould maltases may be essentially identical and their preparations contain secondary substances which are responsible for the noted differences in their properties. Let us assume further that one or both of the preparations contains an excess of secondary substance. Then the activity of a yeast-mould mixture to methyl α-glucoside per unit of yeast present will be greater or less than the activity to this substrate of the yeast component alone, but the activity of the mixture to maltose per unit of yeast present will be greater than the activity of the yeast alone.

3. The yeast and mould maltases may be intrinsically different but one of the preparations may contain accompanying substances which destroy the maltase of the other preparation. Then the activity of a yeast-mould mixture to either methyl α-glucoside or maltose will be that of the resistant component of the mixture alone.

Experiments to be reported below show that yeast-takadiastase mixtures follow the behaviour path predicted for case 3. Hence it seems necessary to conclude that the assumptions set forth for this case apply in fact.

Experimental

Enzyme preparations: 1. Yeast maltase was obtained in solution by extraction of 1 part dry brewery yeast (Givaudon Lavivotte, Lyon) in 10 parts water and toluene with a little magnesium carbonate for 3 hr. at room temperature and then for 20 hr. in the refrigerator before filtering.

2. Commercial takadiastase preparations were kindly provided by Parke Davis and Co., and a laboratory-made preparation of takadiastase (designated as Tn) was kindly provided by Dr M. Frankel.

Substrates: The maltose, sucrose and methyl α-glucoside used as substrates were of pure quality provided by B.D.H.

Methods: The course of hydrolysis was determined by assay of the reducing power of 4 ml. samples withdrawn from the mixtures at different time intervals according to the method of Lehmann Maquenne. Deproteinization was found to be superfluous. Micro-burettes were used for the titration. The mixtures were contained in glass-stoppered Erlenmeyer flasks under toluene. Enzyme + buffer and substrate + buffer blanks were run but gave no significant corrections.

The incubation temperature was 36°.

Experiments reported below are each representative of a series which gave identical results.

Results

(a) *The effect of takadiastase on the hydrolytic activity of yeast maltase solution*

It has already been noted that takadiastase contains an inhibitor for yeast maltase. Several typical experiments showing the nature and extent of the inhibition obtained under different conditions are summarized in the Tables.

Table 1. *Hydrolysis of methyl α-glucoside in the presence and absence of takadiastase by yeast maltase solution*

Mixtures were set up to contain specified quantities of Tn and/or yeast maltase solution, 200 mg. methyl α-glucoside in 20 ml. McIlvaine's citrate phosphate buffer *p*H 6·8, and water to a total volume of 25 ml.

Exp.	Takadiastase ml. 2 % solution in total volume	Yeast ml. solution in total volume	Time of substrate addition	Hydrolysis in % after			
				2 hr.	4 hr.	6 hr.	23 hr.
A	0·00	2·50	Immediately	20	30	36	53
	0·25	2·50	,,	—	28	34	40
	1·00	2·50	,,	20	24	27	31
	2·50	2·50	,,	19	23	23	24
	2·50	0·00	,,	—	—	—	2
B	0·00	2·00	Immediately	9	15	21	37
	1·00	2·00	,,	9	14	17	19
	0·00	4·00	,,	15	25	31	58
	1·00	4·00	,,	15	23	25	40
	1·00	0·00	,,	—	—	—	<1
C	0·00	2·50	After 4 hr.	14	—	—	40
	2·50	2·50	,,	0	—	—	3
	2·50	0·00	,,	0	—	—	2

It will be apparent from the data given that takadiastase, itself almost inert to methyl α-glucoside, does not acquire activity towards the latter in the presence of yeast but actually suppresses hydrolysis of this substrate by yeast α-glucosidase. The suppression takes time: hydrolysis is not materially affected during a period of up to 4 hr. after addition of the inhibitor, but, at the end of this, inhibition sets in and advances rapidly to completion at a rate roughly proportional, within limits, to the square root of the concentration of takadiastase employed (Exp. A). The greater the quantity of yeast present the longer is the time required for the achievement of the full inhibition (Exp. B).

Exp. C of Table 1 shows that a complete inactivation of yeast maltase solution towards methyl α-glucoside is obtained when this is incubated with an adequate quantity of inhibitor in the absence of substrate for a sufficient time interval. The experiment proves that the suppression of maltase activity by the presence of takadiastase is due to a removal of maltase component rather than of substrate from the reacting system.

In several experiments it has been noted that the lag period preceding the onset of the inhibition may be considerably less than 4 hr. Since yeast solution is a variable and rapidly changing preparation it seems easiest to ascribe the variable length of the lag period to changes in the condition of the yeast maltase. Mere incubation of either the yeast or the takadiastase component in buffer at 36° does not, however, abolish the lag, or otherwise change the shape, of the inhibition curve obtained on intermixture.

Some convenient unit for measuring the inhibitor concentration is desirable. The complicated shape of the inhibition curve, however, renders the development of a standard unit difficult at the present stage.

Table 2. *The hydrolysis of maltose by yeast maltase solution in the presence and absence of takadiastase*

An 8% solution of commercial takadiastase was dialysed in cellophane bags against distilled water for 2 days and then tested for inhibitor content. Mixtures were formed to contain specified quantities of yeast solution and/or takadiastase and 10 ml. Sørensen's phosphate buffer *p*H 6·8 in a total volume of 17·5 ml. and were incubated at 36° for 2½ hr. At the end of this time there were added to the mixtures 7·5 ml. of Sørensen's phosphate buffer *p*H 6·8 containing 100 mg. of maltose or methyl α-glucoside.

Yeast ml. in final mixture	Takadiastase ml. in final mixture	Substrate	Hydrolysis in % after 17 hr.
2·50	0·00	Methyl α-glucoside	34
2·50	2·50	,,	4
2·50	0·00	Maltose	24
5·00	0·00	,,	34
0·00	2·50	,,	36
0·00	5·00	,,	60
2·50	2·50	,,	38
5·00	2·50	,,	34

The experimental material presented in Table 2 shows that takadiastase, itself hydrolytically active to maltose, retains this activity on contact with yeast maltase, but at the same time deprives the latter not only of its ability to split methyl α-glucoside but also of its ability to split maltose. Alone both yeast and takadiastase gave a more rapid hydrolysis of maltose when a greater enzyme concentration was used. In mixture an increase in the rate of maltose hydrolysis could be obtained by the use of a greater concentration of takadiastase but not by increasing the concentration of the yeast component within the limits tested. Hence it may be concluded that takadiastase contains a factor which extinguishes the full activity of yeast maltase.

Table 3. *Hydrolysis of sucrose by yeast solution in the presence and absence of takadiastase*

Mixtures were set up to contain 3 ml. 2% Tn solution,[1] and/or 3 ml. yeast solution, and 6 ml. Michaelis acetate buffer *p*H 6·6, in a total volume of 12 ml., and were incubated at 36° for 4 hr. Aliquot samples of the mixtures were then suitably diluted with acetate buffer *p*H 4·5 or 6·6 in which sucrose and methyl α-glucoside were dissolved to give a substrate concentration of 0·4%.

Yeast ml. in 20 ml. of final mixture	Takadiastase ml. in 20 ml. of final mixture	*p*H	Substrate	Hydrolysis in % after ½ hr.	1 hr.	2 hr.	20 hr.
2·00	0·00	6·6	Methyl α-glucoside	—	—	—	55
2·00	2·00	6·6	,,	—	—	—	2
0·00	0·12	4·5	Sucrose	—	<1	1	—
0·12	0·00	4·5	,,	31	56	83	—
0·12	0·12	4·5	,,	31	60	83	—

[1] Tn preparation is relatively poor in sucrase activity and thus peculiarly fitted for testing the effect of takadiastase on the activity of yeast sucrase.

(*b*) *General properties of the inhibitor*

Many of the properties of the inhibitor are those of an enzyme: The inhibitor is completely destroyed when its solution is exposed for a period of 5 min. to the temperature of boiling water.

Table 4. *Effect of heat on the inhibiting potency of takadiastase, and on its maltase activity*

Mixtures were set up to contain 1 ml. 2% Tn solution before and after 5 min. exposure to 100° 14 ml. Sørensen phosphate buffer *p*H 6·8, and 8·0 ml. water containing 100 mg. maltose or 200 mg. methyl α-glucoside, and 2 ml. yeast solution. Parallel mixtures contained no takadiastase, or no yeast, in a total volume of 25 ml.

Enzyme	Substrate	Hydrolysis in % after 24 hr.
Yeast	Methyl α-glucoside	40
Yeast and takadiastase	,,	21
Yeast and heated takadiastase	,,	42
Takadiastase	,,	<1
Heated takadiastase	,,	0
Takadiastase	Maltose	86
Heated takadiastase	,,	80

It is evident from Table 4 that takamaltase activity is only lessened by heat treatment which completely destroys the inhibitor. The inhibitor is non-dialyzable through parchment or cellophane, and has been freed of reducing substances towards Fehling solution by dialysis against distilled water. It is of course soluble in water, and is precipitable from aqueous solution by addition of alcohol, or by addition of acetone to a concentration 60% in the presence of a very little calcium chloride. A crucial test of the enzymic nature of the inhibitor is given by the experiment described in Table 6.

The action of the inhibitor has so far been observed in mixtures buffered by either acetate, citrate-phosphate or phosphate salts. No specific dependence on any of these ions is therefore likely. Table 5 shows that inhibition can be obtained within a range of *p*H values of at least 5·8 to 7·8.

Table 5. *Inhibition of yeast α-glucosidase by takadiastase at different p*H *values*

Mixtures were set up to contain 1 ml. yeast solution, 6 ml. Sørensen phosphate buffer of different *p*H values, 80 mg. methyl α-glucoside, and 1 ml. dialysed 8% commercial takadiastase solution or water in a total volume of 10 ml. Control assays of the final *p*H values were carried out.

	Hydrolysis in % after 16 hr. by	
*p*H	Yeast	Yeast plus takadiastase
7·8	11	6
7·3	25	18
6·8	57	33
6·5	59	40
6·0	59	37
5·8	57	32

The inhibitor's activity shows a marked dependence on temperature: At 17° the action appears to be most sluggish, since mixtures of mould and yeast preparation maintained in buffered solution at 17° for 4 hr. gave a normal inactivation curve, with almost a full lag, when subsequently warmed to 36°.

All tested takadiastase preparations have been found to contain the inhibitor, but the relative inhibiting potencies of different commercial preparations appear to vary considerably.

In order to ascertain whether the inhibitor was used up or modified in the course of its action on yeast maltase, the inhibiting potency of takadiastase preparation before and after incubation with yeast maltase, was measured as in the experiment summarized in Table 6.

Table 6. *Inhibiting potency of takadiastase before and after incubation with yeast maltase*

Mixtures were set up to contain 1 ml. 2% Tn solution, and/or 2 ml. yeast solution, 10 ml. McIlvaine's citrate-phosphate mixture *p*H 6·8, in a total volume of 25 ml. A further mixture contained only 0·66 ml. 2% Tn and buffer in a total volume of 25 ml. The mixtures were kept at 36° for 4 hr., and in the refrigerator for a further 15 hr. To each mixture, warmed again to 36°, there were then added 2 ml. of fresh yeast solution or 2 ml. of water and 10 ml. of McIlvaine's citrate-phosphate buffer *p*H 6·8 containing 200 mg. of methyl α-glucoside. A control mixture contained fresh yeast only as its enzyme component.

Fresh yeast ml. in final mixture	Takadiastase-treated yeast ml. in final mixture	Takadiastase ml. in final mixture	Hydrolysis in % after 3 hr.	Hydrolysis in % after 6½ hr.	Hydrolysis in % after 24 hr.
2·00	0·00	0·00	19	42	70
2·00	0·00	1·00	16	29	44
2·00	0·00	0·66	18	31	49
2·00	2·00	1·00	14	25	42
0·00	2·00	1·00	—	—	2

It will be seen from Table 6 that previous incubation with yeast maltase did not lessen the inhibiting potency of, or abolish the lag period preceding, inhibition by takadiastase of a fresh yeast maltase quantity. It may be seen from the Table that under the conditions of the test a decrease in inhibitor concentration by 1/3 would have been apparent.

Since the properties of the inhibitor are those of an enzyme, the suspicion arose that the inhibitor was identical with maltase or a part of the maltase present in takadiastase. The possible identity of the inhibitor with the full mould maltase was, however, excluded by the finding presented in Table 4 that mould maltase could be obtained free of inhibitor by heat treatment. A fuller consideration of this method of purifying mould maltase is given elsewhere [Hestrin, 1939]. The view of the inhibitor as an enzyme different from the mould maltase was further supported by the observation that the inhibiting potencies of different takadiastase preparations vary independently of their maltase, as well as of their sucrase, activities.

Discussion

Takadiastase has been shown at suitable concentrations and conditions completely to extinguish the activity of yeast maltase solution to either maltose or methyl α-glucoside, though under the same conditions takamaltase itself and yeast sucrase were unaffected. The results of experiments (cf. Tables 1–3) on the specificity of yeast maltase, takamaltase and their mixture are summarized in the following scheme:

Enzyme	Hydrolytic activity: Maltose	Hydrolytic activity: Methyl α-glucoside	Hydrolytic activity: Sucrose
Yeast	+	+	+
Takadiastase-treated yeast	–	–	+
Takadiastase	+	–*	
Yeast-treated takadiastase	+	–	

* Very extended action of high enzyme concentrations on methyl α-glucoside shows a very small hydrolytic activity which is destroyed by heat treatment, but, being very small, may be neglected in any comparison with yeast maltase (Hestrin, 1939).

The qualitatively different behaviours of yeast and mould maltases (=taka-maltase) in respect of the inhibitor in takadiastase may be taken as proof that yeast α-glucosidase (=glucosido-maltase) and mould maltase (=gluco-maltase) are essentially different enzymes and will be found to be different even when they have been freed of all secondary accompanying substances. The experiments reported are therefore compatible with two enzyme systems of maltase classification [Leibowitz, 1925; Leibowitz & Mechlinski, 1926; Haldane, 1930; Tauber, 1934; Leibowitz & Hestrin, 1938; 1939; Hestrin, 1939], but are incompatible with the view that mould and yeast produce essentially one and the same maltase, as Weidenhagen [1933] insists. This conclusion of non-identity refers, however, only to the complete maltase systems involved. It does not bear on the possibility that the different maltases share an identical chemical grouping, and differ only in respect of its carriers [cf. Fodor, 1932; Warburg, 1938].

All known properties of the inhibitor are compatible with the view that the mechanism of the inhibition is enzymic: The inhibitor is non-dialysable, heat-labile and is not used up or materially modified by its action on yeast (cf. Table 6); the inhibition is not instantaneously complete, but takes time, being dependent in its progress on the temperature, on the concentration of the inhibitor and on the quantity of yeast maltase present; the effect is specific and does not extend to yeast sucrase or even to mould gluco-maltase; the inhibitor requires for its action neither the presence of maltose, methyl α-glucoside, nor the specific presence of phosphate, citrate, or acetate salts; the inhibitor is soluble in water, and precipitable from aqueous solution by the addition of alcohol or of acetone with a little calcium chloride. Though the identification of the inhibitor as an enzyme limits the field of choice, it does not narrow it closely. Almost all known kinds of enzyme seem to occur in takadiastase. It has, however, been shown that the inhibitor is not mould maltase (cf. Table 4). Again, the course of the inhibition at different enzyme concentrations, shows that the inhibitor acts after an initial lag period at a rate roughly proportional within limits to the square root of its concentration. This property recalls the Schutz Law for the action of proteases [Northrop, 1924]. Moreover, it has been shown by Tauber & Kleiner [1933] that trypsin inactivates *Q. coli* maltase. Also the known properties of yeast maltase are fully compatible with the view that this maltase involves a protein. Tentatively, it is suggested, therefore, that the inhibitor for yeast maltase in takadiastase is a mould protease. The lag period, which precedes actual loss of activity by yeast solution after exposure to inhibitor, may be explained on the protease hypothesis by assuming that during the lag period the groupings in the yeast maltase micelle which are attacked are not indispensable to its hydrolytic activity.

Pending further knowledge of the nature of the inhibitor, it is proposed to name it *anti-α-glucosidase*. It is perhaps not without significance that anti-α-glucosidase is not the only anti-ferment present in takadiastase preparations. Case & McCullagh [1928] found an anti-myozymase in *Aspergillus eryzae* and believe that this factor is identical with amylase; experiments carried out in this laboratory indicate that takadiastase is able also to inhibit fermentation of glucose by yeast maceration juice. A further examination of these factors is therefore intended.

Summary

1. Preparations of *Aspergillus eryzae* which actively hydrolyse maltose and are almost inert to methyl α-glucoside are not changed in respect of these properties by contact with yeast maltase solution.

LINDA HALL LIBRARY
KANSAS CITY, MO.

2. Yeast solution is deprived of its ability to hydrolyse maltose and methyl α-glucoside by contact with takadiastase.

3. The different behaviours of yeast and mould maltases to the inhibitor in takadiastase is proof that the different properties of these two maltases are a function of differences in the maltases themselves rather than of differences in their accompanying substances. Weidenhagen's identification of mould and yeast maltases is therefore untenable.

4. The inhibiting factor in takadiastase has the properties of an enzyme: It is heat-labile, non-dialysable and is not used up by its action on yeast maltase.

REFERENCES

Case & McCullagh (1928). *Biochem. J.* **22**, 1060.
Fodor (1932). *Ergebn. Enzymforsch., Leipzig*, **1**.
Grassman, Zechmeister, Toth & Stadler (1933). *Liebigs Ann.* **503**, 167.
Haldane (1930). Enzymes, p. 129. London: Longmans, Green & Co.
Hestrin (1939). Dissertation, Jerusalem (in preparation).
Hofmann (1934). *Biochem. Z.* **272**, 417.
Iwanoff, Dodonowa & Tschastuchin (1930). *Fermentforsch.* **11**, 435.
Karstrom (1930). Dissertation, Helsinki.
Kuhn & Munch (1927). *Hoppe-Seyl. Z.* **163**, 1.
Leibowitz (1925). *Hoppe-Seyl. Z.* **149**, 184.
—— & Hestrin (1938). *Nature, Lond.*, **141**, 552.
—— —— (1939). *Nature, Lond.*, **143**, 333.
—— & Mechlinski (1926). *Hoppe-Seyl. Z.* **154**, 64.
Myrback (1931). *Hoppe-Seyl. Z.* **198**, 196.
Northrop (1924). *J. gen. Physiol.* **6**, 723.
Oppenheimer (1936). Die Fermente u. ihre Wirkungen, Supplement, **1**.
Pringsheim, Borchard & Loew (1931). *Hoppe-Seyl. Z.* **202**, 23.
Tauber (1934). *Chem. Rev.* **15**, 99.
—— & Kleiner (1933). *J. gen. Physiol.* **16**, 767.
Warburg (1938). *Ergebn. Enzymforsch. Leipzig*, **7**.
Weidenhagen (1933). *Hoppe-Seyl. Z.* **216**, 255.
—— (1934). *Z. angew. Chem.* **47**, 451.

32. THE SALTING-OUT OF AMINO-ACIDS FROM PROTEIN HYDROLYSATES. A METHOD FOR THE ISOLATION OF *l*-PHENYLALANINE

BY NOEL GARTH BAPTIST AND WILLIAM ROBSON

From the Department of Physiology and Biochemistry, King's College, University of London

(*Received 12 December 1939*)

l-PHENYLALANINE, despite its comparative insolubility in water, is difficult to isolate from protein hydrolysates. Indeed, it would appear from the literature that the only isolation of the fully optically active amino-acid, apart from that claimed by Brazier [1930] which will be discussed below, was that originally carried out by Schulze & Barbieri [1881] when they obtained it from the etiolated sprouts of lupin seedlings. Hitherto it has generally been separated by Fischer's method of ester distillation. The disadvantages of this procedure are too well known to require further comment here and in addition it is universally conceded that even under the best conditions obtainable with high vacuum distillation some 25% at least of the product is racemized in the process. On the other hand whilst synthetic *dl*-phenylalanine can readily be obtained in quantity the resolution of the product is tedious and the yields small.

For some time we have realized the probability that the mixture of amino-acids which separates from a partially or completely neutralized protein hydrolysate saturated with sodium chloride according to the technique of Barnett [1933] or of Hill & Robson [1934] contains phenylalanine in addition to leucine and methionine. Both Barnett's "leucine" fraction and Hill & Robson's "leucine-methionine" fraction, obtained at *p*H 2·2 and 6·0 respectively, give a strong positive Kapeller-Adler reaction for phenylalanine. Moreover, a study of the solubility curves of *l*-phenylalanine, obtained by resolving the synthetic compound, in aqueous and saturated sodium chloride solutions at varying *p*H (Fig. 1), shows that they are practically identical with the corresponding curves for leucine (Fig. 2) as determined by Sano [1926] and by Barnett, a fact which tended to confirm the correctness of our view. For such reasons efforts were directed in the first instance towards the elaboration of a method for the separation of phenylalanine from the leucine-methionine mixture salted-out at *p*H 6·0.

The only method for separating phenylalanine from leucine described in the literature which appeared to us to deserve serious consideration was that of Brazier referred to above. This worker made use of the insolubility of the copper salts of leucine, phenylalanine and aspartic acid to separate these amino-acids from a zein hydrolysate. After regeneration of these acids from their copper salts in the usual way, the aspartic acid was removed by alcohol precipitation of its barium salt in presence of excess baryta. Filtration at this point yielded a solution which, after removal of residual barium, deposited large quantities of leucine on concentration. The remaining solution, which still contained some leucine and most of the phenylalanine, was now boiled with freshly precipitated zinc hydroxide and allowed to cool. The solids which separated from the cold mixture were collected and dried at 100°. The filtrate was evaporated to dryness

and the solids remaining dried at 110°. The two solid fractions thus obtained were separately extracted with cold water, the extracts combined, boiled with more zinc hydroxide, and the previous treatment of the zinc salts repeated until

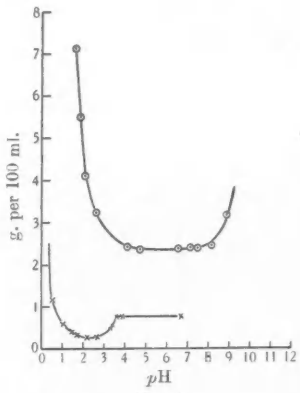


Fig. 1.

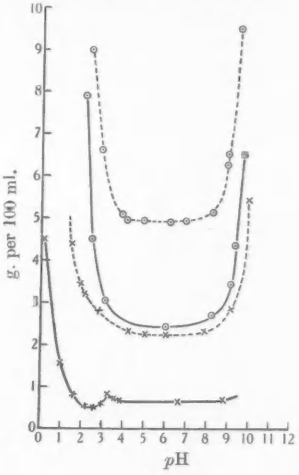


Fig. 2.

Fig. 1. ⊙——⊙——⊙ Solubility of phenylalanine in water. ×——×——× Solubility of phenylalanine in water saturated with sodium chloride.

Fig. 2. ⊙——⊙——⊙ Solubility of leucine in water. ×——×——× Solubility of leucine in water saturated with sodium chloride. ⊙ - - - - - ⊙ - - - - - ⊙ Solubility of methionine in water. × - - - - - × - - - - - × Solubility of methionine in water saturated with sodium chloride.

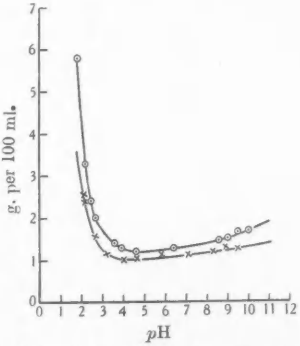


Fig. 3. ⊙——⊙——⊙ Solubility of phenylalanine in water saturated with sodium sulphate. ×——×——× Solubility of leucine in water saturated with sodium sulphate.

the residue obtained was completely soluble in cold water. Brazier claimed that this solution, after removal of the zinc, yielded phenylalanine on evaporation.

Applying Brazier's technique to the "leucine" fraction salted-out from both casein and zein hydrolysates, we have obtained in each case only small quantities of a mixture which consisted chiefly of leucine with smaller proportions of

phenylalanine and methionine as shown by the Kapeller-Adler and nitroprusside reactions. In the light of these findings we have repeated Brazier's process on sulphuric acid hydrolysates of casein and zein, but in neither case have we been able to isolate a pure sample of phenylalanine. It is unfortunate that Brazier gives neither analytical figures, except for nitrogen (Kjeldahl), nor optical data for the material which she reported to be phenylalanine. Since the copper and zinc salts of methionine possess the same order of solubility as those of leucine and phenylalanine, and since, moreover, the methionine content of zein has been estimated by Baernstein [1936] to be 2·2 %, it would appear highly improbable that the phenylalanine isolated in this way is pure, while little reliance can be placed on the nitrogen value of 8·05 % which she quotes for her product (phenylalanine requires 8·48 % N). Her admission, too, that the specimen of phenylalanine which she isolated was difficult to crystallize and showed a tendency to hygroscopy is indicative, in our opinion, of its impurity. Finally, an instance that other workers have been unable to use the method for the purpose described is to be found in the communication of Jukes [1933] on the fractionation of the amino-acids of egg-livetin. On applying it to the amino-acids recovered from the insoluble copper salts obtained from a hydrolysate of this protein, Jukes was unable to separate phenylalanine from the mixture so obtained despite the fact that the Kapeller-Adler colour test showed its presence therein in appreciable quantity.

Another method we originally had in mind for use in attempting to solve this problem was that described by Boyd [1933] by which he separated amino-acids from protein hydrolysates by making use of the different solubilities of their hydantoin derivatives. Actual experiments, however, showed that this valuable method could not be utilized because, apart from the difficulty of avoiding partial racemization of the amino-acids when converting them into their hydantoins, the solubilities of these derivatives of leucine, phenylalanine and methionine are such that no definite separation by fractional crystallization could be achieved.

Attention was finally directed to the possible use of the compounds of phenylalanine with those aromatic substances, e.g. rufianic acid, *p*-benzeneazophenol, picrolonic acid etc. which have from time to time been reported as yielding insoluble crystalline precipitates with this amino-acid. Zimmermann [1930], for example, has reported that rufianic acid forms water-insoluble compounds with phenylalanine, tyrosine and tryptophan, those with alanine, valine, leucine etc. being soluble in water. Under the conditions described by him, we have been unable to obtain a definite crystalline product with either *l*- or *dl*-phenylalanine. The same observation applies to the molecular compound of phenylalanine and *p*-benzeneazo-phenol described by Pfeiffer & Angern [1926]. Of the three reagents mentioned picrolonic acid has found the widest application in this field of work. First introduced by Knorr [1897] as a precipitant for organic bases, the acid was used by Steudel [1903] for the separation of the hexone bases, whilst Mayeda [1907] later described the picrolonates of phenylalanine and tryptophan. Levene & Van Slyke [1912], describing the properties of the picrolonates of several amino-acids, drew attention to the much greater solubility in water of the picrolonate of *l*-phenylalanine over that of *dl*-phenylalanine and demonstrated the use of this reagent for the separation of the components of an artificial mixture of phenylalanine and aspartic acid. Much more recently Bergmann & Niemann [1937] used picrolonic acid to demonstrate the presence of phenylalanine in a digest obtained by the action of papain (proteinase) on cattle-fibrin. Whilst they claimed that a considerable amount of the

amino-acid was liberated, they were able, however, to isolate only 0·3 g. of the 2·5 g. of phenylalanine reported to be present in 100 g. of this protein. In the light of these communications it appeared to us that the possibility of the use of this reagent for our purpose was worth investigating.

Of the three amino-acids, leucine, phenylalanine and methionine, which we now know to occur in the mixture salted-out at *p*H 6·0, the first-named is present in much greater amount than either of the others. From a study of the solubility curves (Figs. 1 and 2) it can be seen that if such a mixture is fractionally crystallized, the solid first recovered will consist wholly of leucine, but there comes a time when phenylalanine will commence to separate from solution. At this stage the mother-liquor will contain approximately equal amounts of leucine and phenylalanine. Accordingly we made up saturated aqueous mixtures of leucine and phenylalanine and of leucine, phenylalanine and methionine as described in the experimental section and to each was added a quantity of picrolonic acid slightly in excess of the amount required to combine with the phenylalanine known to be present. The mixture was boiled to dissolve the reagent, and the resulting solution allowed to cool in the air. When the solution was just warm, i.e. after 2–2·5 hr., the crystals which had separated were collected and twice recrystallized from water. The product thus obtained proved to be pure phenylalanine picrolonate. Moreover, when due allowance is made for the amounts of the picrolonate remaining in solution as determined by Levene & Van Slyke, the amount of the picrolonate actually isolated from the mixtures closely approximated to the theoretical figure.

With these findings as a basis, a method has been elaborated for the separation of *l*-phenylalanine from the "leucine" fraction of amino-acids salted-out at *p*H 6·0 and also from the monoamino-acid fraction obtained by the butyl alcohol extraction of both casein and zein hydrolysates. Briefly it is as follows. The solution of the amino-acids salted-out was concentrated until phenylalanine, as indicated by the Kapeller-Adler colour test, began to separate with the leucine. The mixture was then filtered and the solids, consisting mainly of leucine, washed with a little cold water. The mother-liquor and washings were united, boiled with copper hydroxide and the amino-acids from the insoluble copper salts which separated from the cold solution recovered in the usual way. A Kapeller-Adler estimation carried out on the solution of these amino-acids in water gave an approximate figure for the amount of phenylalanine present. The solution was then boiled down until the concentration of phenylalanine was roughly 2·5%, and picrolonic acid was added to the solution in an amount some 20% in excess of that required to combine with the phenylalanine present. After heating the mixture till all the reagent had dissolved, the resulting solution was allowed to cool in the air (2 hr.) and the long slender crystals of phenylalanine picrolonate which had separated were collected. These were recrystallized twice from minimum amounts of water. The mother-liquor on being left to cool overnight deposited long massive crystals of leucine picrolonate. To obtain the free amino-acid, the phenylalanine picrolonate was dissolved in boiling water, dilute sulphuric acid added until the reaction was definitely acid to thymol blue, and the mixture allowed to cool overnight. The picrolonic acid which separated was then removed by filtration, that remaining in solution being extracted with ether. The aqueous solution, after removal of sulphuric acid with baryta, was evaporated to dryness. Recrystallization of the solid so obtained yielded the characteristic four-sided plates of phenylalanine which after drying with alcohol and ether gave a negative nitroprusside (methionine) reaction, possessed full optical activity and was analytically pure.

From an examination of the solubility curve of phenylalanine in saturated aqueous sodium chloride it would appear that the greater part of this amino-acid present in the protein hydrolysate would be found in the mixture of amino-acids which is salted-out in the processes of Barnett and of Hill & Robson. Yet the amounts of the chemically pure amino-acid actually isolated from such a mixture were only 0·51 % in the case of zein and 0·29 % for casein. Other and unknown factors are apparently involved and as a source of phenylalanine such salted-out mixtures are of little value. On the other hand, application of the new technique to the monoamino-acid fraction obtained by extracting a protein hydrolysate by the butyl alcohol method of Dakin yielded 2·65 % in the case of zein and 1·7 % for casein. These figures are lower than those hitherto recorded. For zein, Osborne & Clapp [1907] recorded 4·8 %; Kapeller-Adler [1932] 5 %; Dakin [1923] quoted 7·2 % although too much reliance cannot be placed on this figure since it is not clear how the author isolated his amino-acid from the undistilled residue of amino-acid esters. For casein the figures are 2·72–3·2 % quoted by Fischer & Abderhalden [1903]; 2·64–3·40 % by Kollmann [1928] and 5 % by Kapeller-Adler, the last figure being the result of a colorimetric determination.

Experimental

Determination of the solubility of l*-phenylalanine in aqueous NaCl*

The *l*-phenylalanine required for these and other preliminary experiments was obtained from the synthetic amino-acid as follows. *dl*-Phenylalanine was synthesized by Erlenmeyer's method as modified by Lamb & Robson [1931] and the product converted into formyl *dl*-phenylalanine according to Steiger [1930]. The resolution of the formyl compound and the liberation of the *l*- and *d*-amino-acids from the resulting products were effected by the methods of Fischer & Schoeller [1907]. The acids obtained yielded the following data on analysis: *l*-phenylalanine $[\alpha]_D^{20^\circ} = -35{\cdot}00^\circ \pm 0{\cdot}20$. Schulze [1885] found $-35{\cdot}3^\circ$. N, 8·40 %. $C_9H_{11}O_2N$ requires 8·48 %. *d*-Phenylalanine $[\alpha]_D^{20^\circ} = +35{\cdot}12^\circ \pm 0{\cdot}20$. Fischer & Mouneyrat [1900] found $+35{\cdot}08^\circ$. N, 8·46 %.

All solubility determinations were carried out in bottles of 50 ml. capacity. The mixture consisting of the solvent and an amount of amino-acid greater than that which would saturate it was shaken mechanically for 8 hr. and then left overnight after which time the solution was filtered directly into a micro-burette. Measured volumes of the solutions were taken for micro-Kjeldahl estimations. The *p*H of the solutions were determined colorimetrically and electrometrically, a double quinhydrone electrode being used for the latter purpose.

Preparation of the picrolonates of phenylalanine, leucine and methionine

dl-*Phenylalanine picrolonate.* *dl*-Phenylalanine (0·1 g.) and picrolonic acid (0·16 g.) were dissolved in boiling water (5 ml.). Rectangular prisms of the picrolonate separated immediately. Water (5 ml.) was added and the mixture again boiled and cooled. The crystals which had separated after 15 min. were collected, washed with much water and dried at 100°. They weighed 0·24 g. (almost theoretical) and melted at 255° (with decomposition) on slowly heating; 214° on rapidly heating. Levene & Van Slyke give 212°.

l-*Phenylalanine picrolonate.* *l*-Phenylalanine (0·083 g.) and picrolonic acid (0·132 g.) were dissolved in boiling water (9·3 ml.). On cooling the solution, the picrolonate immediately commenced to separate as long yellow needles. Water (5 ml.) was added, the mixture re-boiled and set aside to cool. The crystals

which separated were collected, dried first *in vacuo* and then at 100°. The yield of picrolonate was 0·19 g., an almost theoretical figure if allowance is made for the amount remaining in solution (its solubility in water as determined by Levene & Van Slyke is 0·34 g./100 ml.). The picrolonate does not melt when heated slowly; plunged into the bath at 200° it melts with decomposition.

l-*Leucine picrolonate.* *l*-Leucine (0·07 g.) and picrolonic acid (0·132 g.) were dissolved in boiling water (1·5 ml.). On cooling an oil separated. Water (6·5 ml.) was added and re-solution of the oil effected by heating. The picrolonate which separated as long slender rods when the solution was cold melted at 150°. On drying at 100°, the crystals broke down to a powder which on heating melted at 150°, then solidified to melt again at 184° (no decomp.). Levene & Van Slyke give 151°.

l-*Methionine picrolonate.* *l*-Methionine (0·075 g.) and picrolonic acid (0·132 g.) were dissolved in boiling water (3 ml.). On leaving the solution to evaporate slowly in a desiccator over H_2SO_4, the picrolonate was obtained as fine long needles, which melted at 165°. They were recrystallized from water, the product again only separating from solution on its being left in the desiccator for some days. The final product melted at 178–180° after softening at 174°.

The separation of l-*phenylalanine* (*as picrolonate*) *from solutions containing a mixture of* l-*leucine and* l-*phenylalanine and of* l-*leucine,* l-*phenylalanine and* l-*methionine.*

Amino-acids in mixture (A)	Vol. of water in which A was dissolved ml.	Wt. of picrolonic acid added g.	Wt. of phenylalanine picrolonate isolated g.	Theoretical yield less amount remaining in solution g.
0·1 g. P. 0·1 g. L.	10	0·32	0·20	0·23
0·1 g. P. 0·1 g. M.	15	0·20	0·18	0·21
0·1 g. P. 0·1 g. L. 0·1 g. M.	9	0·20	0·215	0·23

P. = *l*-phenylalanine. L. = *l*-leucine. M. = *l*-methionine.

Isolation of phenylalanine from the mixture of amino-acids salted-out at p*H 6·0 from protein hydrolysates*

(*a*) Zein (500 g.; 90 % protein) was refluxed with 20 % HCl (1 l.) for 24 hr. The excess acid was distilled off under reduced pressure. To the syrupy mass of the amino-acid hydrochlorides remaining water was then added and the mixture again concentrated to a syrup, a process which was repeated several times. The final concentrate was dissolved in hot water (2 l.) and to the resulting solution 40 % aqueous NaOH was added until the *p*H of the mixture was 6·0. After being clarified with charcoal, the solution was left to cool overnight. The mixture of tyrosine and leucine which had separated from the cold solution was collected and recrystallized from boiling water. The tyrosine thus isolated weighed 14·5 g. The mother-liquor from this crystallization on evaporation to dryness yielded 56 g. of amino-acids which, as indicated by the Kapeller-Adler colour test, contained no phenylalanine. The original filtrate was further concentrated to approximately 1 l. when NaCl commenced to separate. The resulting mixture was placed in the ice chest overnight and the mass of amino-acids and salt which separated was collected and drained thoroughly by suction. It was then stirred with cold water (200 ml.) and finally sucked dry at the pump.

After being dried at 100°, this salted-out fraction weighed 106 g. and gave only a faintly positive test for chloride. The crude amino-acid mixture so obtained was dissolved in boiling water (1 l.) and the solution chilled overnight. The precipitate (23 g.) which separated was collected and the mother-liquor concentrated to 300 ml., cooled and the amino-acids which crystallized out, weighing 34 g., collected. Further concentration of the mother-liquor to 100 ml. yielded a solution from which was deposited a crop of amino-acids weighing 23 g. and reacting positively to the Kapeller-Adler test for phenylalanine. The filtrate obtained was boiled with an excess of $Cu(OH)_2$, the mixture evaporated to dryness and the residual solids repeatedly extracted with cold water. The Cu salts remaining undissolved were suspended in dilute H_2SO_4, decomposed with H_2S and the filtered solution was freed from $SO_4^{=}$ in the usual way. A Kapeller-Adler estimation carried out on the resulting filtrate showed that it contained 2·6 g. of phenylalanine. After addition of picrolonic acid (4 g.), the mixture was boiled to dissolve the reagent and then allowed to cool. Clusters of slender long needles separated almost immediately and were collected when the solution was just warm. Recrystallization of the product from boiling water yielded a solid which on drying weighed 5·5 g. and melted at 190° with decomposition. The melting point varied somewhat with the rate of heating but the decomposition was quite marked. After drying at 100° for some time, the crystals did not melt on heating strongly but only darkened at 192–194°; plunged into a bath at 192°, the crystals melted immediately with decomposition. Found: $[\alpha]_D^{20°} = 24°$ in alcoholic solution; N, 15·5 %, $C_{19}H_{19}N_5O_7, H_2O$ requires N_2, 15·6 %. The product was therefore considered to be pure phenylalanine picrolonate.

Recrystallization of the last fraction of the crude leucine collected (23 g.) from water provided a mother liquor which, concentrated to 100 ml. and treated with picrolonic acid in the manner described above, yielded a further quantity of phenylalanine picrolonate (1 g.). Hence the total yield of picrolonate obtained was 6·5 g.

To obtain the free amino-acid the picrolonate (2 g.) was boiled with *N* H_2SO_4 (20 ml.) and the solution left in the ice chest overnight. The picrolonic acid which had separated by that time was collected. The filtrate after being extracted twice with ether to remove the last traces of picrolonic acid was freed from sulphate with aqueous baryta in the usual way and the filtered solution concentrated to a small volume under reduced pressure. On cooling the concentrate, a mass of long needles of phenylalanine hydrate separated. Evaporation of an aqueous solution of the solid to a small volume on the boiling water bath led to the separation of the amino-acid in characteristic plates. The yield of the dried amino-acid was 0·72 g. Found: N, 8·40 %. $C_9H_{11}O_2N$ requires 8·48 %. $[\alpha]_D^{20°} = -35·4° \pm 0·7$. The total picrolonate (6·5 g.) would have yielded, on this basis, 2·3 g. of phenylalanine.

(*b*) Casein (500 g.; 84 % protein) was hydrolysed with 20 % HCl (1 l.). The amino-acid fraction salted-out at *p*H 6·0, when treated as described above, yielded phenylalanine picrolonate which after one recrystallization from water weighed 3·3 g., equivalent to 1·2 g. of the free amino-acid.

Isolation of phenylalanine from zein

Zein (100 g. = 90 g. protein) was hydrolysed with a mixture of H_2SO_4 (300 g.) and water (300 g.) for 24 hr. After quantitative removal of H_2SO_4 the solution was concentrated to 1 l. and set aside to cool. During the process tyrosine (3·75 g.) separated and was removed. The mother-liquor was further concentrated to about 250 ml. and the concentrate placed in the ice chest overnight.

The amino-acids (22·85 g.) which had separated in the interval were collected and washed with a little cold water. Phenylalanine was not found in this fraction. The filtrate and washings were combined and then extracted 15 times with warm butyl alcohol when all the phenylalanine, as indicated by the Kapeller-Adler test, had been removed from it. Removal of the butyl alcohol under reduced pressure resulted in the separation of solids weighing 18·8 g. These were dissolved in water, the solution obtained boiled with $Cu(OH)_2$ and the mixture left in the air overnight. The insoluble Cu salts were then collected, washed with cold water and reconverted into the amino-acids in the usual way. A Kapeller-Adler estimation carried out on the resulting solution showed the presence therein of 3·5 g. of phenylalanine. The solution was then concentrated to approximately 100 ml. and treated with picrolonic acid (5 g.). The mixture was boiled to dissolve the reagent and allowed to cool in the air. The solid which had separated whilst the solution was still warm, i.e. after some 2 hr., was collected and recrystallized from water. The phenylalanine picrolonate so obtained, when dry, weighed 6·0 g. The total yield of picrolonate, if the quantities remaining in the various mother liquors (160 ml.) are taken into account, would therefore be 6·54 g.

The picrolonate (6·0 g.), decomposed in the manner already described, yielded 2·2 g. of dry, analytically pure, amino-acid.

Summary

The solubility of *l*-phenylalanine in water and in water saturated with sodium chloride at differing *p*H has been determined.

A new method involving the use of picrolonic acid is detailed for the isolation of phenylalanine from protein hydrolysates.

l-Methionine picrolonate has been prepared and its melting-point determined.

REFERENCES

Baernstein (1936). *J. biol. Chem.* **115**, 25.
Barnett (1933). *J. biol. Chem.* **100**, 543.
Bergmann & Niemann (1937). *J. biol. Chem.* **118**, 781.
Boyd (1933). *Biochem. J.* **27**, 1838.
Brazier (1930). *Biochem. J.* **24**, 1188.
Dakin (1923). *Hoppe-Seyl. Z.* **130**, 159.
Fischer & Abderhalden (1903). *Hoppe-Seyl. Z.* **40**, 215.
—— & Mouneyrat (1900). *Ber. dtsch. chem. Ges.* **33**, 2383.
—— & Schoeller (1907). *Liebigs Ann.* **357**, 1.
Hill & Robson (1934). *Biochem. J.* **28**, 1008.
Jukes (1933). *J. biol. Chem.* **103**, 425.
Kapeller-Adler (1932). *Biochem. Z.* **252**, 185.
Knorr (1897). *Ber. dtsch. chem. Ges.* **30**, 909.
Kollmann (1928). *Biochem. Z.* **194**, 1.
Lamb & Robson (1931). *Biochem. J.* **25**, 1231.
Levene & Van Slyke (1912). *J. biol. Chem.* **12**, 127.
Mayeda (1907). *Hoppe-Seyl. Z.* **51**, 261.
Osborne & Clapp (1907). *Amer. J. Physiol.* **20**, 477.
Pfeiffer & Angern (1926). *Z. angew. Chem.* **39**, 253.
Sano (1926). *Biochem. Z.* **168**, 14.
Schulze (1885). *Hoppe-Seyl. Z.* **9**, 85.
—— & Barbieri (1881). *Ber. dtsch. chem. Ges.* **14**, 1785.
Steiger (1930). *J. biol. Chem.* **86**, 695.
Steudel (1903). *Hoppe-Seyl. Z.* **37**, 219.
Zimmermann (1930). *Hoppe-Seyl. Z.* **188**, 180.

33. CHEMICAL CHANGES IN THE SKIN IN EXPERIMENTAL HYPERTHYROIDISM

By I. ABELIN

From the Department of Physiology, University of Berne, Switzerland

(*Received 13 November 1939*)

The thyroid hormone affects a great number of organs and metabolic processes. The actions of the thyroid on glycogen and fat metabolism, on vitamin utilization etc. are familiar and a relationship between the thyroid hormone and the skin has long been recognized. A soft, warm, slightly sweating skin is among the characteristic symptoms of hyperthyroidism, whilst changes of the skin observed in myxoedema were of historic importance for the development of knowledge of thyroid function. Not much is known, however, concerning the chemical changes in the skin produced by lack or excess of thyroid hormone. The following experiments were carried out as a contribution to knowledge of skin metabolism in experimental hyperthyroidism.

Experimental

White rats of 180–250 g., both female and male, were used for the experiments. They were fed on a diet of milk, bread and raw vegetables. To produce hyperthyroidism both thyroid gland and thyroxine were used. After varying times of treatment with thyroid hormone the animals were killed; as much of the skin as possible was removed, freed from fat, connective tissue and hairs and finely minced. After drying for 24 hr. at 20–24° it was analysed.

Determination of total lipin and total cholesterol

1 g. of dried skin was extracted several times with warm alcohol and then with warm ether until the extracts on evaporation left no residue. The extracts were mixed and evaporated and the residue treated several times with chloroform; the chloroform extracts were made up to 25 ml. and aliquot portions of this solution were used for the determination of total lipin and cholesterol. Several checks showed that extraction of fat and cholesterol by this method was complete. Cholesterol and cholesteryl esters were fractionated by means of digitonin; on the average 42·55 % of the total cholesterol was found to be esterified. Knudson *et al.* [1938] found on the average 47 % cholesterol ester in the skin.

For *determination of water content* the skin was dried at 105° to constant weight.

For *determination of chloride* a carefully weighed sample of skin was ashed with nitric acid after addition of a known quantity of silver nitrate. The excess silver was back-titrated with ammonium thiocyanate.

The experiments on *water imbibition* were carried out on weighed samples of fresh skin freed from hairs. These were immersed 24–48 hr. in buffer solutions of various *p*H, then dried several times with filter paper and weighed.

In some experiments the cholesterol contents of the lungs and the blood were also determined.

All estimations were carried out in duplicate.

The average normal values for total cholesterol, total lipin, water and dry weight of the skin were at first determined on 13 animals of both sexes. The skin contained on the average 261 mg./100 g. of total cholesterol. Knudson *et al.* [1938] found in the skin of the rat an average of 218 mg./100 ml. total cholesterol. The total fat showed considerable variation with an average of 25·82 %. This figure compares favourably with the data of Matthews *et al.* [1935] who found in rat skin an average of 25·72 % total lipin. The ratio total cholesterol : total lipin varies within a relatively small range and averages 0·008.

The water content of normal skin is 55·72 %.

Oral treatment for 5 days with 0·1 g. thyroid daily brings about marked changes in these figures. The total cholesterol content rises to 471 mg./100 g. on the average, i.e. by about +81 %. A similar rise occurs in the total cholesterol : total lipin ratio, which now averages 0·014 as compared with 0·008 in the normal skin. The average water content of the skin rises by 3 %.

Prolongation of the administration of thyroid to 7 days does not alter these figures considerably, but after 15 days the changes become more pronounced. The average cholesterol content of the skin is then 700·29 mg./100 g., whilst the cholesterol : fat ratio rises to 0·023, i.e. almost 3 times the value in normal animals.

Repeated subcutaneous injections of thyroxine have a similar profound effect on the cholesterol content of the skin; 6 rats were injected subcutaneously with 0·75 mg. thyroxine daily for 24 days. The average cholesterol content of the skin was increased to 649·45 mg./100 g., and the cholesterol : fat ratio rose to 8 times the normal value.

Table 1. *Analysis of the skin of normal rats and of rats after administration of thyroid gland or thyroxine (in brackets number of observations)*

	Total cholesterol of dry skin mg./100 g.	Total lipin of dry skin %	Fat content of dry skin %	Cholesterol : fat ratio	Water content of fresh skin %	Chloride content of dry skin mg./100 g.
			Normal			
Highest	363·64	47·33	46·96	0·013	64·21	917·97
Lowest	181·80	13·57	13·39	0·06	50·37	690·58
Mean	261·00 (13)	25·82 (10)	25·56 (11)	0·008 (9)	55·72 (9)	808·91 (4)
			0·1 g. thyroid for 5 days			
Highest	520·50	45·05	44·63	0·017	60·91	992·10
Lowest	423·28	28·26	27·78	0·010	56·33	653·51
Mean	471·51 (4)	34·60 (4)	34·11 (4)	0·014 (4)	58·76 (4)	788·69 (4)
			0·1 g. thyroid for 7 days			
Highest	641·80	43·75	43·32	0·016	62·82	1061·10
Lowest	294·10	19·04	18·74	0·008	46·60	596·26
Mean	437·61 (13)	32·23 (13)	31·80 (13)	0·014 (13)	59·74 (10)	778·83 (5)
			0·1 g. thyroid for 10 days			
Highest	498·67	22·35	21·82	0·025	—	—
Lowest	336·06	20·15	19·67	0·022	—	—
Mean	447·76 (3)	20·89 (3)	20·43 (3)	0·021 (3)	—	—
			0·1 g. thyroid for 15 days			
Highest	752·70	43·25	42·61	0·184	68·41	1185·80
Lowest	629·26	3·77	3·08	0·015	57·24	877·53
Mean	700·29 (11)	20·17 (11)	19·47 (11)	0·023 (11)	62·94 (11)	1057·16 (6)
			0·75 mg. thyroxine daily subcutaneously for 24 days			
Highest	788·17 (1)	35·49 (1)	35·17 (1)	0·123	66·51	1169·00
Lowest	316·60	6·06	5·32	0·009	59·35	875·86
Mean	649·45 (6)	15·65 (6)	15·00 (6)	0·064 (6)	63·78 (6)	961·19 (6)

The chloride and water contents of the skin are closely correlated physiologically. Both values increase in pronounced hyperthyroidism. After 15 days' administration of 0·1 g. thyroid daily the average chloride content of the skin rises from 808·91 mg./100 g. to 1057·16 mg./100 g. The average water content of the skin rises simultaneously by 7 %. The skin of the animals treated for 24 days with thyroxine contains 961·19 mg./100 g. chloride and 63·78 % water on the average, which means an average increase of 152·28 mg./100 g. in the chloride and of 8·06 % in the water content; 5 days' administration of thyroid has little or no effect on the chloride and water contents of the skin.

The results are summarized in Table 1, and those of the cholesterol determinations are represented graphically in Fig. 1.

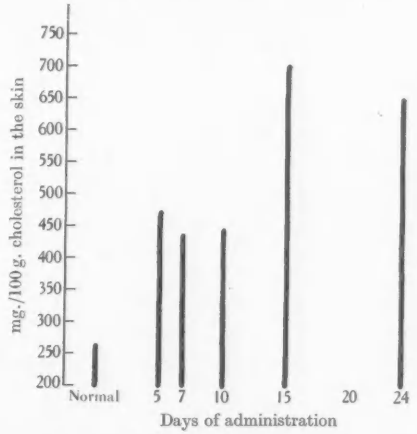


Fig. 1. Average cholesterol content of the skin (mg./100 g.) of normal rats and of rats after administration of thyroid gland or thyroxine.

The rise of the chloride and water content of the skin during hyperthyroidism suggests an increased water-binding power of the constituents of the skin.

To examine this point, swelling experiments were carried out. Carefully weighed samples of fresh skin were immersed in buffer solutions of *p*H 2·92, 3·67, 4·41, 6·10, 8·92, 11·06 and 12·97. Swelling is most pronounced at *p*H 3·67 and 12·97. On the acid side, at *p*H 2·92, normal skin still swells measurably. At this *p*H the swelling of the skin of the hyperthyroidic animal, however, is so marked that complete gelation occurs and reliable determinations become impossible. Each swelling experiment lasted 24–48 hr. The figures below illustrate the results obtained.

Average increase of weight (%) *of skin samples during 24–48 hr. swelling in buffer solutions*

	*p*H 3·67	*p*H 12·97
Animal No. 11 (normal)	+ 29·82	+ 55·65
Animal No. 12 (normal)	46·68	53·26
Animal No. 13 (normal)	48·00	80·71
Animal No. 35, after 14 days' administration of thyroid	+157·5	+ 96
Animal No. 36, after 14 days' administration of thyroid	140·0	192·5
Animal No. 37, after 14 days' administration of thyroid	112·5	101·0

Analyses were also made of the cholesterol of the lungs and blood of normal and hyperthyroidic rats. The lungs of groups of 6 animals gave the following results in mg./100 g.: (*a*) normal animals: 461·90, 465·22, 392·17, 420·70, 486·43, 337·10; average: 427·10; (*b*) animals after 16 days' administration of 0·1 g. thyroid daily: 469·82, 479·17, 550·06, 457·15, 485·19, 480·76; average: 487; (*c*) animals after 24 days' administration of 0·75 mg. thyroxine daily: 387·05, 423·80, 419·20, 439·00, 285·30, 359·87; average: 384·10.

The changes are thus slight and irregular, and indeed scarcely outside the range of physiological variation.

The cholesterol of human blood in cases of hypo- or hyper-function of the thyroid has been studied by several workers. The data in the literature are divergent, but the majority of authors agree that in cases of hypo-function the cholesterol content of the blood is increased; in hyperthyroidism, irregular variations have been observed. A decrease of blood cholesterol is certainly not as constant a finding in hyperthyroidism as is an increase in myxoedema [cf. Schmidt & Hughes, 1938]. In the experiments reported here experimental hyperthyroidism did not cause marked changes in the cholesterol content of the blood.

The cholesterol content of the blood of the normal rats used in these experiments averaged 40 mg./100 ml. After 7 days' administration of 0·1 g. dried thyroid daily the following values (mg./100 ml.) for blood cholesterol were found: 53·13, 44·94, 39·47, 35·84, 41·44; average: 42·97. After 2 weeks of the same treatment the values of blood cholesterol content were: 42·12, 47·26, 43·02, 53·19, 44·94, 39·47, 35·84, 41·44; average: 43·40.

Discussion

Among the characteristic symptoms of hyperthyroidism is an increased liberation of energy and correspondingly an increased loss of heat. As is well known this loss occurs mainly by means of radiation, heat conduction and evaporation of water from the skin. These regulating mechanisms are used to a large extent during thyrotoxicosis.

Cardiac output is also increased during hyperthyroidism, the small blood vessels being dilated. By the increased circulation in the skin more heat is transported to the surface of the body, thus facilitating the loss of heat to the surroundings. As shown in the present investigation, administration of thyroid gland also diminishes the fat content of the skin. Fat is a poor heat conductor and, under physiological conditions, protects the organism against undue loss of heat. Its decrease during hyperthyroidism will therefore facilitate the increased loss of heat.

Another process of importance for the increased loss of heat through the skin is the evaporation of water in the form of both visible and invisible perspiration. The increased cholesterol content in the skin during hyperthyroidism may play a part in this connexion, since cholesterol enhances the imbibition of water by the tissues. Apart from the absolute cholesterol content the cholesterol : fat ratio is particularly important; this ratio (called "coefficient lipocytique" by French authors [cf. Mayer *et al.* 1938]) actually rises during hyperthyroidism up to 10 times its normal value. The skin can then take up considerably larger quantities of water, the evaporation of which will contribute to the heat loss. Increases in both water content and swelling power of the skin after prolonged thyroid administration are, as shown above, actually demonstrable.

Water and chloride metabolism usually change in parallel fashion. In the present experiments this rule is followed, the increase of water content of the

skin being accompanied by a corresponding increase of chloride content. These changes, however, occur only during pronounced hyperthyroidism; administration of small quantities of thyroid hormone scarcely affects the normal values, as shown in Table 2.

Table 2

	Chloride content of the skin mg./100 g.	Water content of the skin %
Normal	808·91	55·72
After 5 days' administration of thyroid powder	788·70	56·90
After 7 days' administration of thyroid powder	973·54	58·76
After 15 days' administration of thyroid powder	1057·16	62·94
After 24 days of thyroxine	1005·53	63·78

The source of the great quantities of cholesterol which appear in the skin during experimental hyperthyroidism is unknown. Whether this material is mobilized or newly synthesized can only be decided by further experiments. The cholesterol content of the blood, however, did not change during thyroid administration in the present experiments, which does not favour the assumption of cholesterol mobilization from other organs; moreover, in the lungs no decrease of cholesterol could be demonstrated. It is, however, possible that the cholesterol comes from other organs, perhaps from the liver, and the possibility of new formation of cholesterol in the organism (particularly in the liver) is not excluded.

The skin contributes not only to the excretion of water, but also of iodine. During experimental hyperthyroidism the skin of rats is considerably richer in iodine than is that of normal animals [Abelin & Wehren, 1940], and it gives off more iodine to the surroundings than the normal skin. Whether the changed chemical composition of the skin, particularly the increased cholesterol content, is related to its iodine content is however not yet clear.

Summary

The skin of rats after administration of thyroid gland or thyroxine over a considerable period shows the following changes in its chemical composition: its cholesterol, chloride and water contents rise, while the neutral fat content diminishes. The skin of rats in experimental hyperthyroidism has an increased water-binding power.

By facilitating the loss of heat from the surface of the skin these chemical changes are related to heat regulation.

The low fat content enhances the heat conduction and the high cholesterol content (up to 300 % of the normal values) favours the water imbibition of the skin and the succeeding evaporation of water. Possibly also loss of iodine through the skin is favoured.

No definite changes occur in the cholesterol content of the lungs and the blood during thyroid administration.

REFERENCES

Abelin & Wehren (1940). *Arch. int. Pharmacodyn.* **64**, 91.
Knudson, Sturges & Bryan (1938). *J. biol. Chem.* **126**, 547.
Matthews, Newton & Bloor (1935). *J. biol. Chem.* **108**, 145.
Mayer, Schaeffer & Terroine. Cf. Cristol (1938). Précis de chimie biologique médicale, Paris.
Schmidt & Hughes (1938). *Endocrinology*, **22**, 474.

34. SOLUBLE RESERVE-CARBOHYDRATES IN THE LILIIFLOREAE

By NILS GRALÉN and THE SVEDBERG

From the Institute of Physical Chemistry, University of Uppsala, Sweden

(*Received 30 December 1939*)

Carbohydrates are often stored in plants to be used in subsequent metabolic processes. Cell membranes are permeable to the low-molecular carbohydrates, and therefore the carbohydrates in storage organs are usually polymerized. Sometimes the polymerization goes so far that the carbohydrates become insoluble in water, e.g. starch, but in other instances the stored carbohydrates remain soluble although appreciably polymerized. The best-known soluble polysaccharide is probably inulin, which is built up exclusively of fructose molecules. Fructose seems to be a rather common unit in soluble polysaccharides. Johansson [1889] found several inulin-like carbohydrates in the Gramina. Parkin [1899] found what he called inulin (i.e. fructose-yielding polysaccharides) in the bulbs and tubers of several monocotyledons, sometimes together with starch. Monosaccharides other than fructose also build up soluble polysaccharides. Glucose, mannose and galactose are all found as constituents of polymeric soluble carbohydrates (lichenin, "Konjak"-mannan, polygalactans and others).

During an investigation of soluble high-molecular carbohydrates in plants [Svedberg & Gralén, 1938] it was found that the sap from the bulbs of different species of the family Liliaceae gave distinct and characteristic sedimentation diagrams on ultracentrifugal examination. This fact led to a more exhaustive investigation of different species and genera of Liliaceae and the closely related plant families.

Material and its preparation

The monograph of Engler & Prantl [1930] has been used as the reference to botanical systematics.

The work has been limited to the plant families Liliaceae, Amaryllidaceae and Iridaceae. These three families belong to the group Liliifloreae, which is divided into the subgroups Juncineae (rush plants), Liliineae (lily plants), and Iridineae (iris plants). In general the rush plants have only small dry rhizomes and, consequently, they were considered not to be suitable objects for this investigation. The Liliineae include the families Liliaceae and Amaryllidaceae together with some smaller families containing mostly tropical species. Iridaceae is the only family of Iridineae.

About 75 different species, representing a selection from the three families mentioned, have been investigated. A large proportion of the material was obtained from the Botanical Garden of Uppsala. Most of the other material was bought from Tubergen Ltd., Haarlem, Holland.

From the biological point of view it is certainly of greatest interest to study the polysaccharides in as nearly as possible the native state. The preparation was made as follows. The bulb or the tuber for investigation was peeled and grated or ground in a meat-grinder in order to break up the cell structure. The grater has been used almost exclusively. There is less risk of cracking the starch grains, which might cause solution of the inner part of the grain, the amylose. The

grated bulb was mixed with some distilled water to recover more of the dissolved material, especially if the bulb did not contain much juice. In most cases an amount of water equivalent to the weight of bulb material was added. After mixing for 2 min. with a glass rod the sap was pressed out through a filtering cloth and centrifuged free from starch and other undissolved material. In order to avoid enzymic breakdown the preparations were always made immediately before the ultracentrifuging. The time from the beginning of the preparation until the start of the centrifuge experiment usually did not exceed half an hour.

In many instances it was found that besides the high-molecular substances the juice contained low-molecular materials (salts, monosaccharides etc.) in such large amounts that they influenced the sedimentation diagram very much. To reduce this effect the solutions were dialysed for 2 days at $+4°$ against $0{\cdot}2\,M$ NaCl, which was used as a standard solvent throughout the investigation. The salt concentration used is certainly sufficient to eliminate electrostatic effects, owing to charges on the sedimenting large molecules. During dialysis some precipitate often formed, but most of the high-molecular substances were left in solution, and the same diagram peaks have always been obtained from the fresh and the dialysed solutions if allowance is made for the low-molecular material in the freshly prepared solutions. The precipitate formed has not been further investigated. It is probable that the precipitation was due to decreased solubility brought about by the lowering of the temperature and of the salt concentration.

Methods

The construction and operation of the ultracentrifuge is described by Svedberg & Pedersen [1940].

The ultracentrifuge experiments were carried out with the scale method of Lamm [1937; 1940]. The centrifugal force amounted to 300,000–350,000 times gravity at the speed used (65,000–70,000 r.p.m.). The cell thickness was 12 mm. Violet or green monochromatic light from a Hg arc lamp has been used for the photographic observations, depending on the colour of the solution. Most of the solutions were almost colourless, allowing use of the violet light. Some plant juices show a tendency to grow dark soon after the cell structure has been destroyed, owing to oxidative formation of some coloured substance by exposure to the air. It was possible to dialyse away part of the colour. Some of the remaining colour sedimented in the ultracentrifuge. The visible sedimentation column slowly grew brighter during the experiment, but a sharp colour boundary was never obtained, and it was never possible to assign the colour to any of the observed high-molecular substances (carbohydrates or proteins).

The diagrams presented here are ordinary scale diagrams from ultracentrifuge experiments. The abscissa is the distance from the centre of rotation (in mm.). To the left is the meniscus of the solution at a distance of 58–60 mm. from the centre of the rotor, and to the right is the bottom of the cell about 72 mm. from the centre. The ordinate represents the scale line displacement (in mm.), which is proportional to the concentration gradient in the cell. Each maximum of the curve represents a sedimenting boundary. The sedimenting substance is to the right of the boundary, and to the left is the pure solvent from which the sedimenting substance has disappeared. A broad peak means a diffuse boundary, a high and narrow peak means a sharp boundary. For each diagram the time after reaching full speed (in min.) and the scale distance (in mm.) are noted. The scale line displacement is proportional to the scale distance.

The continuous curves are obtained from dialysed solutions, the broken curves from the native juices. All diagrams are obtained with 0·2 *M* NaCl as reference scale. The low-molecular material in the juice always sediments a little from the meniscus, but its diffusion is so large that it does not give rise to any peak in the diagram. It is seen only as a raising of the curve towards the meniscus. The effect of the low-molecular material is clearly visible on Fig. 24 (*Narcissus poetaz*). The same diagram illustrates the fact that the sedimenting material moves faster in a dialysed solution. The low-molecular material in the juice decreased the sedimentation rate by increasing the density and viscosity of the solvent.

Colorimetric analyses for carbohydrates according to Dische [1930] have been made on practically all of the dialysed solutions investigated. Kjeldahl analyses for nitrogen have also been made. All of the solutions contained both carbohydrate and N but there were extreme variations in the quantities. The N found analytically has been taken as an indication of protein. Several of the solutions containing large amounts of N were tested by heating for 5 min. in boiling water. Coagulation always took place, and analyses of the filtrates showed that most of the N had disappeared, whereas the carbohydrate content was not changed. This suggests that the assumption that the N represented protein is roughly correct.

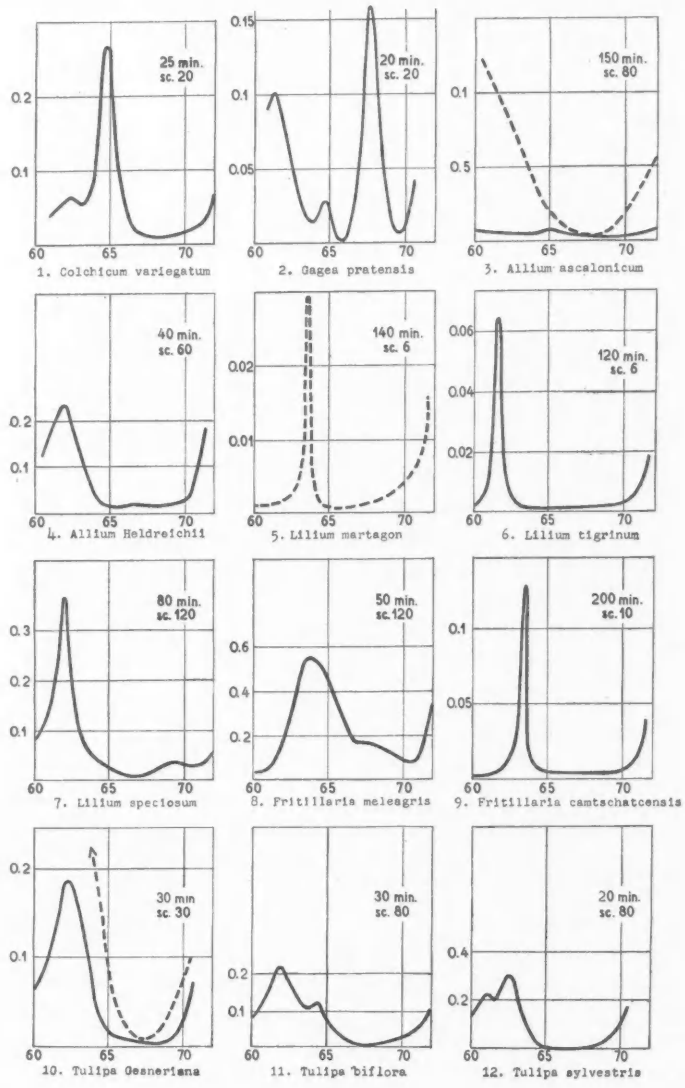
Table 1

		Concentration in g. per 100 ml.					
			By analyses				
				Nitrogen			
Species	Sedimentation constants	Calculations from the diagram	Carbohydrate	N	% prot. = 6 × N	Nature of high-molecular material	Fig.
LILIACEAE:							
Melanthioideae:							
Colchicum vernum	3·3; 5·4	0·58; 2·29	0·11	—	—	Protein	(1)
C. autumnale	3·3; 6·3	0·12; 0·92	0·11	0·172	1·03	Protein	(1)
C. variegatum	3·1; 6·0	0·18; 1·00	0·11	0·196	1·17	Protein	1
Allioideae:							
Gagea pratensis	1·1; 7·7; 12·5	0·34; 0·18; 0·78	0·56	0·184	1·10	c.h. + prot.	2
Allium ascalonicum	(7·7)	0·06	0·09	0·009	0·05	—	3
A. Heldreichii	1·6	0·31	0·21	—	—	c.h.	4
Brodiaea uniflora	3·1	3·91	8·8?	0·184	1·10	Mostly c.h.	(14)
Lilioideae:							
Lilium candidum	0·7	Not calculable	1·6	0·030	0·18	c.h.	(5, 6)
L. bulbiferum	0·9	0·96	1·0	0·048	0·29	c.h.	(5, 6)
L. martagon	1·3	0·25	—	—	—	c.h.	5 (6)
L. tigrinum	0·8	0·46 uncertain	0·89	0·020	0·12	c.h.	(5), 6
L. speciosum	1·1; 5·7	0·64; 0·39	0·56	0·067	0·40	c.h. + prot.	7
L. Henryi	1·1	0·38	—	—	—	c.h.	(5, 6)
L. Maximowiczii × *Willmottiae*	1·0	0·84	1·31	0·038	0·23	c.h.	(5, 6)
Fritillaria meleagris	2·3; 6·2	0·73; 0·21	0·22	0·224	1·34	Mostly prot.	8
F. imperialis	2·4	0·50	0·06	0·141	0·84	Mostly prot.	(8)
F. camtschatcensis	1·2	0·53	0·76	0·052	0·31	c.h.	9
F. pluriflora	1·1	0·25	0·28	0·018	0·11	c.h.	(14)
Tulipa praecox	2·9; 6·0	0·26; 0·13	0·12	0·056	0·34	c.h. + prot.	(11)
T. Gesneriana	3·4	1·10	0·44	0·180	1·08	Mostly prot.	10
T. biflora	3·4; 6·4	0·39; 0·21	0·11	0·071	0·42	c.h. + prot.	11
T. sylvestris	3·4; 5·7	0·17; 0·24	0·30	—	—	c.h. + prot.	12
Erythronium dens canis	3·2	0·56	0·07	—	—	Protein	(10)
Calochortus albus	2·9	0·11	0·03	0·012	0·08	Protein	(10)

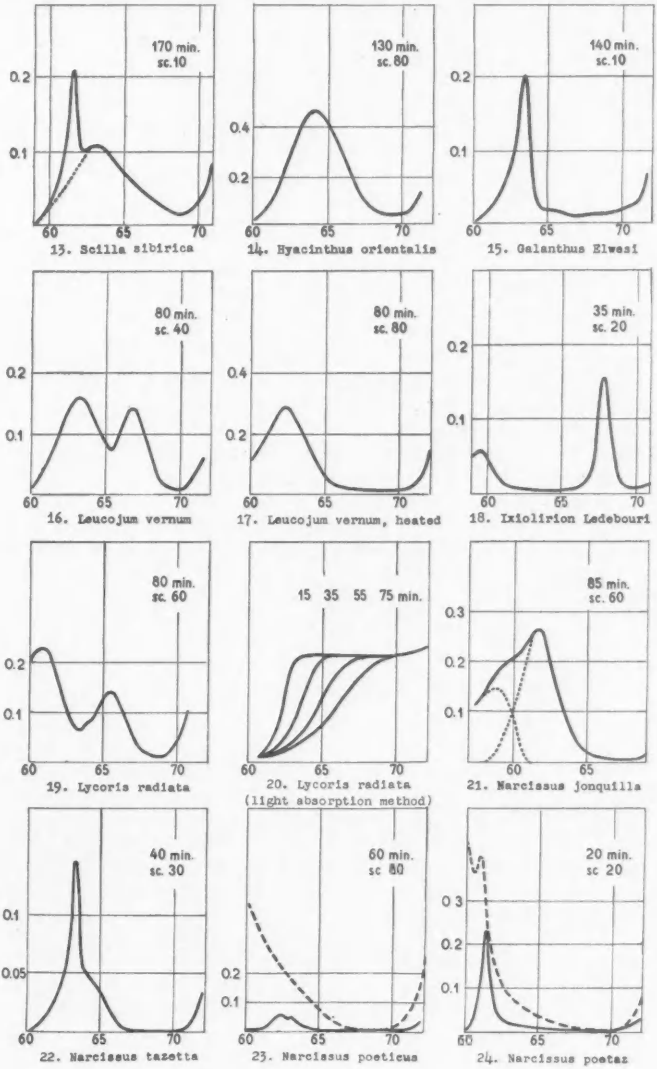
Table 1 (*cont.*)

Species	Sedimentation constants	Concentration in g. per 100 ml.				Nature of high-molecular material	Fig.
		Calculations from the diagram	By analyses				
			Carbo-hydrate	Nitrogen			
				N	% prot. $=6 \times N$		
Scilloideae:							
Dipcadi serotinum	1·3	0·45	0·61	0·013	0·08	c.h.	(14)
Scilla sibirica	1·1; 1·7	0·6; 2·4	3·7	0·108	0·65	c.h.	13
Camassia esculenta	1·9	0·88	1·04	0·020	0·12	c.h.	(14)
Ornithogalum comosum	2·4	0·46	0·73	0·007	0·04	c.h.	(14)
O. umbellatum	1·5	2·0	3·55	0·012	0·07	c.h.	(14)
Chionodoxa gigantea	1·2	0·46	0·58	0·020	0·12	c.h.	(14)
Puschkinia scilloides	1·2; 1·7	0·90; 0·24	1·39	0·042	0·25	c.h.	(13)
Hyacinthus orientalis	1·6	1·7	1·8	0·037	0·22	c.h.	14
Muscari botryoides	1·7	1·7	2·06	0·005	0·03	c.h.	(14)
M. comosum	3·2	0·92	1·62	0·021	0·13	c.h.	(14)
Asparagoideae:							
Polygonatum multiflorum	1·3	2·75	2·95	0·014	0·09	c.h.	(14)
Paris quadrifolia	2·8	0·09	0·22	0·015	0·09	?	(5, 6)
AMARYLLIDACEAE:							
Amaryllidoideae:							
Galanthus nivalis	4·3	0·14	0·16	0·032	0·19	?	(10)
G. Elwesi	1·2	0·46	0·78	0·014	0·09	c.h.	15
Leucojum vernum	1·3; 4·3	0·71; 0·52	0·45	0·126	0·76	c.h. + prot.	16, 17
L. aestivum	1·6; 3·6	0·20; 0·19	0·22	0·042	0·25	c.h. + prot.	(16)
Amaryllis Belladonna	—	—	0·04	0·008	0·05	?	(3)
Crinum Moorei	1·0	0·16	1·1?	0·009	0·06	?	(3)
Ixiolirion Ledebouri	1·2; 12·2	0·32; 0·53	0·61	0·079	0·47	c.h. + prot.	18
Lycoris radiata	1·0; 3·6	0·13; 0·09	0·22	0·048	0·29	c.h. + prot.	19, 20
Narcissus pseudonarcissus	1·4	0·50	0·47	0·018	0·12	c.h.	(5, 6)
N. cyclamineus	1·1	1·01	1·11	0·051	0·31	c.h.	(14)
N. juncifolius	1·3	0·55	0·64	0·030	0·18	c.h.	(5, 6)
N. jonquilla	2·7; 4·0	0·42; 0·99	0·21	0·207	1·24	c.h. + prot.	21
N. tazetta	1·8	0·69	0·37	0·087	0·52	c.h. + prot.	22
N. poeticus	1·6; 1·8	0·10	0·11	0·008	0·05	c.h. + prot.	23
N. poetaz	1·9	0·34	0·30	0·019	0·11	c.h. + prot.	24
N. Bulbocodium	1·2	0·37	0·36	0·018	0·11	c.h.	(5, 6)
N. incomparabilis	1·4; 2·4	0·56; 0·48	0·56	0·073	0·45	c.h. + prot.	(7)
N. Leedsi	1·2	0·25	0·30	0·039	0·23	c.h.(+ prot.)	(5, 6)
Hypoxidoideae:							
Alstroemeria aurantiaca	—	—	0·16	0·013	0·08	?	(3)
IRIDACEAE:							
Crocus reticulatus	3·1; 6·2	0·39; 0·20	0·07	0·094	0·56	(c.h.) + prot.	25
C. sativus	2·7; 5·9	0·36; 1·74	0·68	0·249	1·49	c.h. + prot.	26
C. speciosus	3·0; 6·5	0·35; 0·24	—	—	—	(c.h.) + prot.	(25)
Freesia refracta	3·1	2·30	0·20	0·287	1·72	Protein	(10)
Sparaxis tricolor	2·9; 5·4; 17·8	1·14; 0·66; 1·38	0·64	0·484	2·90	c.h. + prot.	27
Gladiolus segetum	2·9; 6·5	0·73; 0·92	0·16	0·161	0·97	Mostly prot.	(28)
G. communis	2·8; 6·5	0·50; 0·77	—	—	—	Mostly prot.	28
G. byzantinus	2·9; 6·9	1·02; 0·23	0·16	0·169	1·01	Mostly prot.	29
G. grandis	3·2; 6·9	0·58; 0·60	0·07	0·138	0·83	Mostly prot.	(28)
G. cuspidatus	3·2; 6·7	1·09; 0·41	0·08	0·210	1·26	Mostly prot.	(29)
G. anatolicus	2·7	0·31	0·02	0·061	0·36	Protein	(17)
G. Colvillei roseus	3·0; 6·3	0·84; 1·11	0·15	0·195	1·17	Mostly prot.	(28)
Moraea tricuspis lutea	4·5	2·9	0·27	0·307	1·84	Protein	(10)
Iris squalens	8·0	0·02	0·02	0·009	0·05	?	(3)
I. reticulata	0·9; 8·7	0·72; 0·59	1·14	0·152	0·91	c.h. + prot.	30

LINDA HALL LIBRARY
KANSAS CITY, MO.

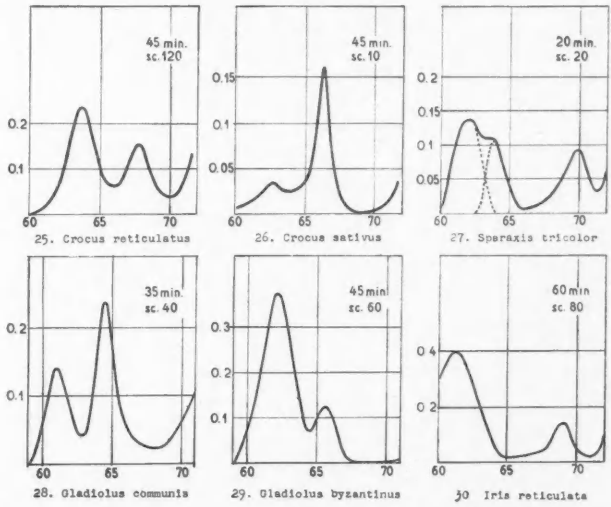


1. Colchicum variegatum
2. Gagea pratensis
3. Allium ascalonicum
4. Allium Heldreichii
5. Lilium martagon
6. Lilium tigrinum
7. Lilium speciosum
8. Fritillaria meleagris
9. Fritillaria camtschatcensis
10. Tulipa Gesneriana
11. Tulipa biflora
12. Tulipa sylvestris



13. Scilla sibirica
14. Hyacinthus orientalis
15. Galanthus Elwesi
16. Leucojum vernum
17. Leucojum vernum, heated
18. Ixiolirion Ledebouri
19. Lycoris radiata
20. Lycoris radiata (light absorption method)
21. Narcissus jonquilla
22. Narcissus tazetta
23. Narcissus poeticus
24. Narcissus poetaz

The concentration of the sedimenting material has been calculated from the sedimentation diagrams, assuming a refractive index increment dn/dc of $1{\cdot}5 \times 10^{-3}$, which is an approximate value for carbohydrates (cf. p. 246). The comparison of the analyses and the calculations from the curves very often gives information about the nature of the sedimenting material. The method is, however, not very accurate. It was very often not possible to remove all turbidity from the solutions used for analyses. This would give too high analytical values, because in the ultracentrifuge the turbidity always disappeared at a relatively low speed (15,000–20,000 r.p.m.). Sometimes it was difficult to determine the exact



position of the base line of the sedimenting curves, especially when the peak was broad and even elongated over the whole cell (see, for instance, Fig. 14, *Hyacinthus orientalis*). The base line position affects the concentration calculation to a considerable extent. In spite of the difficulties mentioned, the procedure has given good results in many instances.

For every species at least two ultracentrifuge experiments have been made, one on the freshly prepared juice, one on the dialysed solution. The results are collected in Table I and in Figs. 1–30. The Table contains the sedimentation constants[1] (s_{20}) obtained from the dialysed solutions, reduced to pure water and the temperature 20°. The concentration calculations from the diagrams are given in the next column. If several peaks are obtained in the same diagram, the sedimentation constants are given in order starting with the lowest, and the concentrations for the corresponding peaks in the same order. The results of the analyses for carbohydrate and N are also reported. The protein content has been calculated, assuming it to be 6 times the N content. In one column is reported the nature of the sedimenting substance, as indicated by the analysis. The last column gives a reference to the number of the corresponding diagram. If the number is put in parenthesis, the diagram referred to was obtained not from the species in question but from another of the same sedimentation type.

[1] Sedimentation constants are given in units of 10^{-13}.

Discussion of results

Only a few species failed to yield a noticeable amount of dissolved high-molecular material in the bulb juice. *Allium ascalonicum*, the cultivated onion, had considerable low-molecular material in the juice, but after dialysis practically nothing was visible (Fig. 3). Another species of the genus *Allium*, *A. Heldreichii*, yielded a small amount of a high-molecular carbohydrate (Fig. 4). This raises the question as to whether the cultivation has caused the breakdown of a pre-existent polysaccharide in *A. ascalonicum*.

There is an analogy in the genus *Tulipa*. The cultivated tulip, *Tulipa Gesneriana*, contains a large amount of low-molecular material hiding possible peaks. After dialysis, however, a large peak appears (Fig. 10). The analyses show a high N content but only a small amount of carbohydrate, and therefore it is probable that the peak represents a protein. The same diagram has been obtained from different varieties of *T. Gesneriana*. The wild tulips here investigated, *T. sylvestris*, *T. praecox*, *T. biflora*, all show two peaks (Figs. 11, 12). Precipitation by alcohol and redissolving (which was not complete) gave a solution which was practically N-free. The sedimentation diagram of that solution showed absence of the faster moving peak of the original diagram. Obviously the protein has been denatured by the alcohol, and, consequently, the carbohydrate of these species belongs to the slower component. The alcohol-precipitate from *T. Gesneriana* could not be redissolved in water in any appreciable amount.

The *T. Gesneriana* type diagram with a large protein constituent was found with some of its near relatives, *Erythronium dens canis* and *Calochortus albus*. Other, more distantly related plants, also had a high protein content and gave similar diagrams. This was found for *Colchicum* (Fig. 1, fam. Liliaceae), *Freesia* and *Moraea* (fam. Iridaceae). For *Colchicum* and *Moraea*, however, the sedimentation constants were higher.

Where there is only one peak in the diagram, it is generally rather easy to decide from the analyses whether it is caused by a carbohydrate or a protein. If two or more peaks appear and the analyses show presence of both N and carbohydrate, the question of identification of the substances with the peaks of the diagram arises. It is sometimes possible to draw conclusions from the relative proportions of the peaks. It is obvious that the largest peak in *Lilium speciosum* (Fig. 7) represents a carbohydrate and the smaller peak, which sediments more rapidly, is a protein. In *Crocus sativus* (Fig. 26) the faster, large peak is the protein, while the low concentration of high-molecular carbohydrate appears in the diagram as the smaller, slow peak.

If the difference between the concentrations is not so large, however, the method is not accurate enough to give any sure results. The precipitation with alcohol gave results for the *Tulipa* species. Another method that was used successfully was the heating of the solutions for 5 min. in a boiling water bath. In most cases the protein coagulated but the carbohydrate was left in solution. The ultracentrifuging of the remaining solution always showed that one of the peaks disappeared as a result of the heating. The two diagrams of *Leucojum vernum* (Figs. 16 and 17) were obtained before and after heating. The same procedure has been applied to *Narcissus incomparabilis*, *Crocus sativus* (Fig. 26), *Gladiolus byzantinus* (Fig. 29) and *Iris reticulata* (Fig. 30), and the results always showed that the faster-sedimenting peak represented a protein, whereas the carbohydrate was slower. In the case of *Gladiolus byzantinus*, the slower peak also contained protein as well as the carbohydrate, this being shown by a

marked decrease in that peak after heating, whereas the faster peak disappeared completely.

Ultracentrifuging in ultraviolet light is probably the mildest possible treatment which can be given the solution and be expected to yield information about the nature of the sedimenting substances. The proteins have a strong light absorption band around 260 mμ, whereas the carbohydrates do not absorb until at a very much lower wave-length. Chlorine and bromine filters have been used for obtaining a suitable wave-length region for the protein absorption band [Svedberg & Nichols, 1926]. The applicability of the method is shown by the example of *Lycoris radiata* (fam. Amaryllidaceae). The first diagram (Fig. 19) is the ordinary scale diagram. The second one (Fig. 20) shows the photographic blackening curves from the ultraviolet light absorption experiment. The sedimentation constants calculated from the former are 1·0 and 3·6. The latter gives $s_{20}=3{\cdot}3$ which is in good agreement with the faster peak in the scale diagram, while the slower peak cannot be detected in the absorption diagram and so represents the polysaccharide.

Other methods for separating proteins and carbohydrates have not been used, but there are certainly several mild methods that could be applied. Electrodialysis would probably precipitate the proteins which presumably are mainly globulins, while the carbohydrates would stay in solution. Separation centrifuging [Tiselius *et al.* 1937] could possibly be used if the sedimentation constants are widely different. Electrophoresis [Tiselius, 1937; 1938] would presumably give separation at a suitable *p*H. The proteins are certainly carriers of electrical charges, but the carbohydrates are more likely non-electrolytes [cf. Seibert *et al.* 1938].

In some cases it has not been possible to decide whether the peaks are caused by proteins or carbohydrates. The diagram of *Gagea pratensis* (Fig. 2), for instance, has no less than three peaks, one of them with the very high $s_{20}=12{\cdot}5$. It is noticeable that *Gagea* yields such a detailed diagram while *Allium*, very closely related systematically, gives no indication of high-molecular substances.

The very high sedimentation constant ($\sim$12) of the fastest *Gagea*-peak is found also with *Ixiolirion Ledebouri* (Fig. 18, fam. Amaryllidaceae). It is very characteristic for both these species although they are systematically widely different. A still higher sedimentation constant, $s_{20}=17{\cdot}8$, was obtained with *Sparaxis tricolor* (Fig. 27, fam. Iridaceae), which contains at least three different high-molecular constituents, as indicated by the dotted lines in the diagram. Heating gave no information about *Ixiolirion* or *Sparaxis*, because the proteins did not precipitate completely.

It seems impossible to draw parallels between the centrifuge diagrams and the larger divisions of systematics. Similar diagrams are found with material from different families and widely different diagrams are obtained with material from the same family, showing that the carbohydrate storage is not similarly organized within the same family. In the smaller units, the genera, the diagrams are, however, usually very similar. There are exceptions even to this rule, and they sometimes have a special interest.

Fritillaria meleagris and *F. imperialis* yield similar diagrams (Fig. 8) and contain almost exclusively proteins as the high-molecular constituents of their bulb-juices. *F. camtschatcensis* yields a different diagram (Fig. 9), a very sharp, slowly moving peak, representing a carbohydrate. The same picture is given by almost all *Lilium* species that have been examined (Figs. 5, 6). It is known that *F. camtschatcensis* is more closely related to the genus *Lilium* than are the other *Fritillaria* species [Buxbaum, 1937]. (Engler & Prantl [1930] assign it to a sub-

genus called *Liliorhiza*.) On the basis of the present results it would even be preferable to assign it to the genus *Lilium* itself.

The subfamily Scilloideae of Lilioideae yielded a typical diagram. It was obtained from the genera *Dipcadi*, *Camassia*, *Ornithogalum*, *Chionodoxa*, *Hyacinthus* and *Muscari* (Fig. 14). It shows a wide peak that spreads out more and more during the sedimentation. The sedimentation constant varied considerably for different species, but it is possible that most of the variation was due to differences in concentration which affect the sedimentation rate. The analyses showed that the sedimenting material was a carbohydrate.

An unusual diagram was obtained from *Scilla sibirica* (Fig. 13). It showed two peaks, both of which were from carbohydrates judging by the analyses. Heating the solution to 100° did not change the character of the diagram, even though some precipitation occurred which lowered the N content. It is possible to resolve the diagram into its two constituents as indicated on the diagram. The faster-moving peak is broad and of the same type as those in the diagrams from the other Scilloideae. The slower peak has a different appearance, being sharper and much higher, although the concentration, proportional to the area of the peak, is lower than for the more rapidly moving carbohydrate. The sharp peak is very similar to those obtained from the *Lilium* bulbs (Figs. 5, 6).

The diagram from *Puschkinia scilloides* was similar to that of *Scilla sibirica*. It is obvious that the external similarities between the two species, which have given the *Puschkinia* species its name, correspond to an internal resemblance in the physico-chemical organization of the plants.

Carbohydrates of the same type as those of the genus *Lilium*, resulting in the very sharp boundary in the sedimentation diagram (Figs. 5, 6), are found also in the family Amaryllidaceae within the genus *Narcissus*. All the juices giving this diagram are very slimy. There are also slimy bulbs, however, which give different diagrams (*Hyacinthus* and others, especially in the subfamily Scilloideae).

The genus *Narcissus* shows different types of composition of the high-molecular substances in the bulb juices. The "*Lilium*-peak" (Figs. 5, 6) is obtained from *N. pseudonarcissus*, *N. juncifolius*, *N. Bulbocodium* and *N. Leedsi*. *N. incomparabilis* also shows it but combined with a faster-sedimenting substance which has proved to be a protein (precipitates on heating). *N. cyclamineus* yields the *Hyacinthus*-diagram (Fig. 14). *N. jonquilla* contains considerable protein, probably in both the components of the diagram (Fig. 21). *N. tazetta* has the very sharp *Lilium*-peak (Fig. 22). *N. poeticus* contains much low-molecular material, and the high-molecular substances are not visible in the diagram until after dialysis (Fig. 23). It gives two small, rather diffuse peaks. *N. poetaz* (Fig. 24), a hybrid *N. poeticus* × *N. tazetta*, has a large amount of low-molecular material like *N. poeticus*, but the sharp carbohydrate-peak of *N. tazetta* is distinct. Obviously it has acquired properties in this respect from both its parents. The ultracentrifuge method here opens a new field for genetics, and it will be of great interest to investigate the way in which the properties of reserve-carbohydrate storage are inherited. Some research in this direction has been started.

Most of the soluble carbohydrates found in bulb juices can be arranged into two classes as regards their sedimentation behaviour. The first one is typically represented by the *Hyacinthus* (Fig. 14) and the second one by the *Lilium* bulbs (Figs. 5, 6). The *Hyacinthus* polysaccharide gives a very broad peak, spreading almost over the whole sedimentation column, while the *Lilium* polysaccharide

sediments with a very sharp boundary. The sedimentation constants for both are of the same order of magnitude, 1–2, but the differences in the sedimentation behaviour indicate quite different physico-chemical properties.

The *Hyacinthus* type most probably represents a mixture of different molecular sizes, varying continuously according to some distribution law. The limits are presumably rather wide, as judged by the sedimentation curves. The hydrolysis products of the carbohydrates have not been examined, but it is probable that these polysaccharides are in some instances identical with the polyfructans found earlier especially in the subfamily Scilloideae of the family Liliaceae [Parkin, 1899]. Parkin pointed out that most species of this subfamily contain "inulin" together with starch. It should be noted that the inulin in the juices pressed from the tubers of *Inula helenium*, *Dahlia coccinea* and *Helianthus tuberosis* (all belonging to the family Compositae), gave sedimentation diagrams of the same type and a sedimentation constant of the same magnitude.

The second type of polysaccharide, the *Lilium*-type, is of more interest because of its peculiar sedimentation diagram. It shows, as mentioned, a very sharp boundary for the sedimenting material, indicated by a high and sharp peak in the diagram. A protein or other globular molecule with the same low sedimentation constant would have so high a diffusion that no maximum in the curve would be obtained, because it would never leave the meniscus at the same centrifugal force. In this case, however, there is scarcely any diffusion indicated in the centrifuge cell. The same phenomenon has been observed by earlier investigators on other substances, especially cellulose derivatives [Kraemer & Nichols, 1940; Signer, 1940]. The sharpness of the peak indicates a thread-like shape of the molecules. These molecules are considerably hindered in their motion by each other, and the effect is greater the higher the concentration. The sedimentation constant is therefore higher in a more dilute solution. If some molecules are left behind, they, being in a more dilute solution, sediment at a more rapid rate. On the opposite side of the boundary where the concentration increases, the molecules will move more slowly. These effects counteract the diffusion which would normally be expected, and cause the sharpening of the boundary.

Purifying the polysaccharide of *Lilium speciosum* by precipitation with alcohol and redissolving in water did not change the sedimentation diagram or the sedimentation constant for a given concentration. The relation between the concentration and the sedimentation constant is given by Fig. 31. A diffusion experiment was carried out on the purified material according to Lamm's method [1937]; cf. Polson [1939]. Using the formula

$$D = \frac{x_1^2 - x_2^2}{4t \log_e y_2/y_1}$$

where D is the diffusion constant, t the time, x the distance from the original boundary and y the height of the concentration gradient curve at the point x, it is possible to calculate diffusion constants from different parts of the curves obtained. D showed a drift along the curve, which proves inhomogeneity. From the sedimentation experiment one would expect the carbohydrate to be homogeneous, i.e. contain molecules of only one size, but the diffusion experiment shows that such a conclusion is not justified. Near the original boundary the lowest values of the diffusion constant are obtained because the large molecules diffuse more slowly. With increasing distance from the original boundary the diffusion constants increase, because the curve is mainly determined by the smaller molecules there. The drift in D for the *Lilium* polysaccharide was from

about 4·0 to 5·4.[1] This means a still greater variation in the true diffusion constant, because the smallest value here given was obtained from the highest part of the curve where both the larger and the smaller molecules are effective in determining the gradient, while the largest D was obtained from the low part where the curve is almost exclusively determined by the smaller molecules. For the molecular weight a mean value of 20,000 was obtained, assuming the partial specific volume to be 0·64 and using the formula

$$M = \frac{RTs}{D(1 - V\rho)}$$

[Svedberg & Pedersen, 1940], where M is the molecular weight, R is the gas constant, T the absolute temperature, s the sedimentation constant (taken at zero concentration), D the diffusion constant, V the partial specific volume and ρ the density of the solvent (water). The value 20,000 corresponds to about 125 units of monosaccharides.

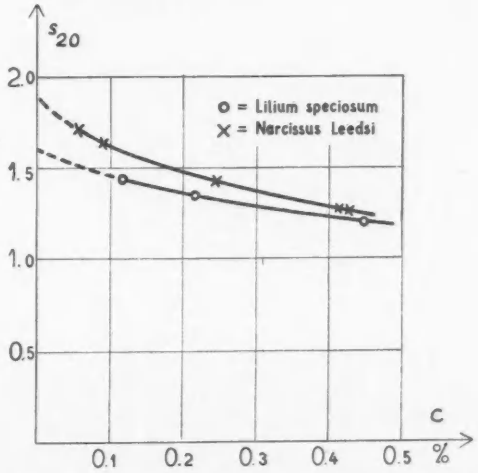


Fig. 31. Relation between concentration and sedimentation constant of purified carbohydrates from *Lilium speciosum* and *Narcissus Leedsi*.

Another polysaccharide with the same sedimentation behaviour was prepared from the bulbs of *Narcissus Leedsi* Southern Gem (from Tubergen Ltd.). The water-extract from the bulbs was precipitated twice by alcohol, the redissolved polysaccharide was filtered through filter paper pulp, dialysed and electrodialysed. Kjeldahl analyses showed that the N content was less than 1 % of the dry material. The sedimentation constant depends on the concentration as shown by Fig. 31. The polysaccharide was very stable; it could be heated to 100° for 30 min. in neutral solution, or dried *in vacuo* over P_2O_5 and redissolved without any change of s_{20}. In the dry state it was a transparent, fairly brittle film, while the solution was faintly opalescent. The partial specific volume in the solution was 0·640, which is appreciably lower than the figures for proteins (in general 0·73–0·75 [Svedberg & Pedersen, 1940], but in good agreement with

[1] Diffusion constants are given in units of 10^{-7}.

those for cellulose (0·64 [Stamm, 1930]). The refractive index increment dn/dc (c in %) was $1{\cdot}46 \times 10^{-3}$, which corresponds closely to the values for other carbohydrates, both mono- and poly-saccharides. The specific rotatory power was $\alpha_D = -42°$, which is of the same order of magnitude as for inulin ($-39°$, $-40°$). The polysaccharide was hydrolysed by boiling for 2 hr. in 4% H_2SO_4, and a preliminary study of the hydrolysis products was made. Seliwanoff's reaction [Roe, 1934] with resorcinol showed that only about 20% of the material was a ketose (probably fructose). Mannose could be demonstrated in a small amount (~10%) by its phenylhydrazone (M.P. 190°). The rest of the carbohydrate (70%) was probably glucose, because it yielded a large amount of phenylglucosazone (M.P. 204°).

Diffusion experiments have shown that the *Narcissus Leedsi* polysaccharide was polydisperse, the variation of D being from about 2 at the top of the curve to about 4 at the bottom. No dependence on concentration could be demonstrated. A mean value of 50,000 was obtained for the molecular weight, if calculated with the sedimentation constant at zero concentration, 1·9. Viscosity measurements gave $\eta_{sp}/c = 47$, where c is expressed in base molarity, according to Staudinger [1932]. This would give a Staudinger proportionality factor $K_m = 9 \times 10^{-4}$, which is comparatively high and gives still more evidence of the thread-like shape of the molecules.

No completely homogeneous substance has been found among the polysaccharides investigated. Nature seems to prefer a mixture of polymeric homologues, even if the polydispersity is definitely different for different systems. For some of the natural soluble products the heterogeneity is less than for artificial solutions of polysaccharides such as cellulose and starch and for synthetic products like polystyrenes. The reason for the polydispersity of polymeric carbohydrates is possibly that the differences between a polysaccharide molecule and its nearest higher and lower homologues are very small. The elongation of the chain with one or a few units will not change the properties of the molecule much, particularly as the largest part of the molecule is far away from the new linkage. Another class of high-molecular compounds, the globular proteins, have a different type of organization. They consist of different amino-acids in regular proportions, the molecules have a more spherical shape, and there is a strict organization within each molecule. Therefore the molecule must be totally built up according to its plan before it is suited to fill its function in the vital processes. When it is fully completed, it has no use and no place for any more amino-acids, and so all the protein molecules of the same kind are equal in size and shape. If a foreign amino-acid were linked to the complete protein, it would change the organization and shape of the molecule more than a monosaccharide would alter the polysaccharide molecule to which it became attached. The polysaccharide is suitable for its functions even if it consists of relatively few monosaccharide units. Its constituents are more homogeneous than the widely different amino-acids of the protein. Very often the monosaccharide units are the same through the whole molecule, and even if they are not, the differences among the hexoses are not large. Therefore the polysaccharide can be built up continuously without marked changes in the qualitative properties of the molecule. There is certainly some regulation of the size of the molecules, probably of enzymic nature, but it need not definitely restrict the number of monosaccharide units in every molecule, and, consequently, there is a variation within wide or narrow limits.

From the biological point of view it would be of great interest to know if the soluble polysaccharides constitute any transition form between the solid poly-

saccharides (starch, cellulose etc.) and their building units, the monosaccharides. Certainly some of the carbohydrates studied here are reserve materials in the plants and so have some function of their own, but it is very possible that others serve only as an intermediate stage in the breakdown of starch or other reserve-carbohydrates for transport in the plants or in the building up of the solid, structural polysaccharides, the prototype of which is cellulose. Further investigations are proceeding to give a more detailed outline of the physico-chemical properties of some of the soluble polysaccharides found in plant juices. It is hoped that this research will contribute to the solution of the question of the functions of these substances in the plants.

Summary

1. An ultracentrifugal study of the water-soluble high-molecular materials contained in the bulb juices of about 75 species of the families Liliaceae, Amaryllidaceae and Iridaceae has been made.

2. The different species yield widely different sedimentation diagrams. They contain proteins and polysaccharides of different properties and in different proportions.

3. There is generally a pronounced similarity among the species of the same genus with regard to the content of high-molecular material. The seven *Lilium* species investigated, for instance, all contain a large amount of dissolved polymeric carbohydrate, and they all give the same type of sedimentation diagram. Outside the genera, the differences are large even within the same family. Species of *Fritillaria* and *Tulipa*, which belong to the same subfamily as *Lilium*, generally contain in solution more protein than carbohydrate and yield diagrams widely different from the *Lilium* diagrams. One *Fritillaria* species, however, *F. camtschatcensis*, which is closely related to *Lilium*, gives a diagram of the *Lilium* type.

4. Among the carbohydrates found there are two classes, distinguished by their sedimentation behaviour. One of them, the *Hyacinthus* type ($s_{20} \sim 1$–2), found especially within the subfamily Scilloideae of the family Liliaceae, gives a broad, diffuse boundary, indicating a marked polydispersity. The second one, the *Lilium* type, obtained with the genera *Lilium* (Liliaceae) and *Narcissus* (Amaryllidaceae), although having a sedimentation constant of the same magnitude, yields a very sharp boundary, which is probably due to the thread-like shape of the molecules.

5. Two individuals of the *Lilium* type polysaccharide, from the species *Lilium speciosum* and *Narcissus Leedsi*, have been purified and subjected to a more detailed physico-chemical investigation. The sedimentation constants extrapolated to zero concentration were found to be, for the *Lilium* species 1·6, and for the *Narcissus* species 1·9. Diffusion experiments in combination with these figures gave mean molecular weights of 20,000 and 50,000 respectively.

6. The polydispersity of the soluble polysaccharides has been discussed in comparison with the monodispersity of the globular proteins.

The expenses connected with this work have been defrayed by grants from the Rockefeller and the Wallenberg Foundations.

The authors wish to express their thanks to the directors of the Botanical Garden of the University of Uppsala, Prof. E. Melin and later Prof. J. A. F. Nannfeldt, for their courtesy in permitting a collection of bulbs and tubers to

be made. We are greatly indebted to Dr N. Hylander of the Botanical Garden for his kind help and advice in selecting species and for valuable suggestions in questions of systematics.

REFERENCES

Buxbaum (1937). *Bot. Arch.* **38**, 367.
Dische (1930). *Mikrochemie*, **8**, 4.
Engler & Prantl (1930). *Die natürlichen Pflanzenfamilien*, Band **15** *a*. Leipzig.
Johansson (1889). *Svensk. Vet. Ak. Handl.* N.F. **23**, No. 2.
Kraemer & Nichols (1940). In The Ultracentrifuge (see Svedberg & Pedersen).
Lamm (1937). *Nova Acta Soc. Sci. upsal.* **10**, IV, No. 6.
—— (1940). In The Ultracentrifuge (see Svedberg & Pedersen).
Parkin (1899). *Philos. Trans.* B, **191**, 35.
Polson (1939). *Kolloidzschr.* **87**, 149.
Roe (1934). *J. biol. Chem.* **107**, 15.
Seibert, Pedersen & Tiselius (1938). *J. exp. Med.* **68**, 413.
Signer (1940). In The Ultracentrifuge (see Svedberg & Pedersen).
Stamm (1930). *J. Amer. chem. Soc.* **52**, 3047, 3062.
Staudinger (1932). Die hochmolekularen organischen Verbindungen. Berlin.
Svedberg & Gralén (1938). *Nature, Lond.*, **142**, 261.
—— & Nichols (1926). *J. Amer. chem. Soc.* **48**, 3081.
—— & Pedersen (1940). The Ultracentrifuge. Oxford.
Tiselius (1937). *Trans. Faraday Soc.* **33**, 524.
—— (1938). *Kolloidzschr.* **85**, 129.
—— Pedersen & Svedberg (1937). *Nature, Lond.*, **140**, 848.

35. A NOTE ON THE CARDIAC INOSITOL OF THE SHEEP, PIG AND OX

By L. B. WINTER

From the Department of Physiology, University of Sheffield

(*Received 4 January 1940*)

THE work to be described was undertaken with the object of investigating the nature of the combined inositol shown to be present in the heart muscle of the dog [Winter, 1934]. It was hoped that a more readily available source of heart muscle might be used, and experiments were carried out with sheep's heart, with the surprising result that combined inositol could not be found: nor could any be found in the heart muscle of the pig and ox.

EXPERIMENTAL

In the case of the sheep and pig, hearts were brought in ice from the slaughter house; no such precaution was taken with the ox hearts. Ventricular muscle was always used, minced in an ice-cold mincer, and two 35 g. portions were heated at 100° with 35 ml. of 10% KOH under reflux, one for 30 min. and the other for 5 hr. The work was begun before the publication by Young [1934] of his volumetric method for estimation of inositol in tissues, and it was decided to continue with the method of isolation and weighing used by the writer, with the following modification. The basic Pb precipitate was washed twice with weak NH_4OH (about 0·08 *N*), separated by the centrifuge (5 min. at 9000 r.p.m.), suspended in 50 ml. of water, and decomposed with H_2S. The PbS was removed by filtration, well washed with hot water, and the filtrate concentrated to dryness *in vacuo* at 35–40°. About 30 ml. of ether were added to the flask and poured off after standing a for a day at room temperature. 7·5 ml. of water were placed in the flask and, when the solid was dissolved, 90 ml. of strong alcohol. The flask was allowed to stand at room temperature for a day (any longer interval involving risk of loss of inositol), the precipitate was removed by the centrifuge and 25 ml. of water were added to the alcoholic solution, after which the inositol was precipitated as the Ba compound.

In the following Table are given the weights of inositol obtained in each duplicate experiment, expressed as mg. per 100 g. of fresh heart muscle:

			Sheep				
Heated ½ hr.	75·0	39·2	43·6	32·6	48·1	43·9	74·8
Heated 5 hr.	62·6	35·4	56·9	23·7	42·8	63·2	65·8
			Pig				
Heated ½ hr.	31·4	28·6	98·1	60·0			
Heated 5 hr.	30·5	18·6	75·6	38·5			
			Ox				
Heated ½ hr.	74·0	73·2	54·8	72·5			
Heated 5 hr.	51·3	59·0	46·4	71·0			

It will be seen that in the 13 experiments 11 show a decrease of inositol after the longer heating with KOH, and two show an increase. Possible reasons for the latter may be experimental error, or unequal distribution of inositol in

different parts of the heart muscle. The decrease in inositol found in the majority of the experiments suggested that the substance may not be so stable to alkali as is generally believed. In the first paper on the subject [Winter, 1934] the writer accepted the statements in the literature, e.g. Tollens [1914], that inositol, like glycogen, is very resistant to alkali. The matter has been tested by heating 40 mg. inositol in 40 ml. of 5% KOH under reflux at 100° for 5 hr., and comparing the amount recovered with that in parallel experiments in which the alkaline solution was not heated, and was neutralized immediately after addition of the inositol. There was marked destruction of inositol by the alkali; the decrease (average of 4 experiments) was 43·7%. It is not to be expected that the destruction of inositol has been as great in the heart muscle experiments, since the effective strength of alkali would be much reduced by the buffering action of the proteins and the products of their hydrolysis. Some destruction must, however, have taken place, and it is not safe to conclude that combined inositol is absent from the heart muscle of the sheep, pig and ox, since the figures obtained may be the resultant of destruction of free inositol and liberation of some from combination. The complete absence of combined inositol would be surprising in view of the findings with the dog's heart: a reasonable interpretation of the above results is that the combined inositol of the cardiac muscle of the sheep, pig and ox must be much less than that of the dog. It might be argued that since the dog's heart muscle was added to boiling KOH within a minute or two of the death of the animal, while in the case of the slaughter-house material a much longer interval occurred, much of the combined inositol, if present, was broken down by tissue enzymes and free inositol liberated. This is unlikely to be the reason for the failure to demonstrate any appreciable amount of combined inositol in the heart muscle of the sheep, pig and ox, for it was shown that the combined inositol of the dog's heart was similar in amount, whether the tissue was dealt with immediately or was allowed to suffer survival changes for 90 min. at body temperature. The results described above also render it unlikely that the combined inositol of the dog's heart is a substance such as phytin, derived from plant sources in the food and of no metabolic significance; if this were so, it would be expected that more of the substance would be present in the muscle of herbivorous animals such as the sheep and ox, or the omnivorous pig, rather than the carnivorous dog.

Summary

1. It has not been possible to detect combined inositol in the heart muscle of the sheep, pig and ox.

2. Inositol is destroyed to a considerable extent when boiled with 5% aqueous potassium hydroxide.

REFERENCES

Tollens (1914). Handbuch der Kohlenhydrate, p. 664.
Winter (1934). *Biochem. J.* **28**, 6.
Young (1934). *Biochem. J.* **28**, 1435.

36. INFLUENCE OF ALKALINE TREATMENTS AND PRETREATMENTS IN HEMICELLULOSE PREPARATION

By ISAAC ARTHUR PREECE

From the Chemistry Department, Heriot-Watt College, Edinburgh

(*Received 12 January 1940*)

The method for the preparation of hemicelluloses originally proposed by Norris & Preece [1930] involves a preliminary treatment of the tissue material with 0·5% ammonium oxalate solution for the removal of pectin and water-soluble substances followed by treatment with boiling 50% ethyl alcohol-1% NaOH solution with the intention of diminishing contamination of the subsequent preparation with lignin. Hemicelluloses are then removed from the residue by solution in 4% aqueous NaOH.

It was pointed out later by Preece [1931] that the alcoholic-NaOH treatment may remove from the tissue a notable amount of furfuraldehyde-yielding material. The material so removed was tentatively designated "lignosaccharide"; that from box-wood showed marked resistance to hydrolysis, but otherwise there was found no reason to distinguish it from other structural hemicelluloses which, themselves, show varying degrees of resistance to the action of boiling dilute acid.

Alcoholic-NaOH pretreatment in the preparation and determination of hemicelluloses has been criticized by Norman [1935; 1937, 1, 2]. Thus, it is claimed that the amount of material so extracted varies according to the concentrations of alcohol and alkali used. The objection is a sound one. Again, use of the term "lignosaccharide" is objected to on the ground that insufficient difference of properties is shown between this material and other hemicelluloses to justify the introduction of a further complication into the already confused nomenclature of these substances. This objection is perhaps less sound for, if it is borne in mind—as has been expressly emphasized [Preece, 1931]—that the term implies a structural hemicellulose prepared by the method described, there can be little objection to its use until the relationship between this and other hemicellulose fractions is more clearly understood.

In an investigation of the hemicelluloses of the hop flower [Angell & Norris, 1936] the alcoholic-NaOH pretreatment was retained, the authors stating that furfuraldehyde-yielding substances could not be detected in the extract. Norman [1937, 1], however, states that the treatment involves a loss of such materials, even though furfuraldehyde itself cannot be obtained from the extract. Figures supporting this contention are presented, and the statement is made that the only true criterion of preservation of furfuraldehyde is a proof that the tissue residue shows no reduction in furfuraldehyde yield. This suggests interesting possibilities, with alkali treatment apparently resulting in a destruction of furfuraldehyde-yielding substances. The following hitherto unpublished figures for a 9–10-year-old sample of lilac wood certainly appear to support the idea of such destruction. The analysis was carried out by the method

of Preece [1931], and the tissue residue after removal of "combined hemicellulose" represented approximately 57·5% of the original dry wood; it gave 6·22% of its own dry weight of furfuraldehyde, corresponding to a yield on the original dry wood of 3·58% of furfuraldehyde. The figures of Table 1 are

Table 1

Furfuraldehyde from substances soluble in:	%
(i) 0·5% Ammonium oxalate	
(*a*) Pectin	0·37
(*b*) Non-pectin	0·46
(ii) Alcoholic-NaOH	0·72
Furfuraldehyde from:	
(iii) Free hemicellulose	4·37
(iv) Combined hemicellulose ...	1·16
(v) Tissue residue	3·58
Total	10·66

calculated on the original dry wood; however, the furfuraldehyde yield found directly was 12·44%. The discrepancy between the two totals is surprisingly high, and it cannot be readily accounted for unless degradative changes have occurred during extraction. It is impossible that as much as 14% of the total furfuraldehyde could be represented by normal "extraction losses".

Turning now to the extraction of other hemicellulose fractions, it may be asked whether it is allowable to follow the extraction with NaOH in the cold with a boiling extraction with this solvent. The original justification of such a proceeding lies in figures such as those for box-wood shown in Table 2, these

Table 2

	On dry wood	
Furfuraldehyde from:	Lilac wood %	Box-wood %
(i) Ammonium oxalate extract	0·83	0·26
(ii) Lignosaccharide and free hemicellulose	5·09	4·34
(iii) Xylan associated with crude cellulose	7·22	5·11
Total	13·14	9·71
Total furfuraldehyde found directly	12·44	11·86

being re-calculated from the results of the analysis already published [Preece, 1931]. It is apparent from the box-wood figures that 2·15% of furfuraldehyde remains unaccounted for; the aim of the boiling NaOH treatment was to endeavour to trace the reason for this though, to be sure, similar summations for other woods (see, for example, the figures included for lilac) exceed the total found directly, which gives obvious evidence of overlapping. Part at least of the box-wood deficit may, in view of what has already been said, be due to the destructive effect of the alkali. However, apparent justification for the treatment is found in the composition of the materials isolated from box-wood as "combined hemicellulose"; thus, the products were of polyuronide hemicellulose type, similar to those extracted by cold 4% NaOH.

It is clear that further study is required of the effects on yield and composition of extractives at varying temperatures and of various concentrations, and of the suggested possibility of a degradative effect of the treatments. An examination of some aspects of the problem is described below.

Experimental

Furfuraldehyde determination

The influence of the various treatments employed is first measured by noting the changes induced in the furfuraldehyde yield. The difficulties inherent in the furfuraldehyde determination are well known, and a valuable résumé of its problems is provided by Dorée [1933]. Again, Angell *et al.* [1936] point out certain difficulties of interpretation of results, and state that it is inadmissible to use the Kröber conversion factors when alcoholic extraction of the phloroglucide precipitate is employed. Circumstances having precluded the use of the distillation method described by these authors, the normal distillation procedure was followed in the present work, but alcoholic extraction of the phloroglucide precipitate was omitted. Furfuraldehyde (f) was calculated from the weight of phloroglucide (p) using the formula: $f = 0{\cdot}5185\,(p + 0{\cdot}0052)$. It may be noted that the weights of phloroglucide here involved are uniformly of the order 0·06–0·11 g.

Whilst the results presented are possibly somewhat few in number for statistical analysis, it is very desirable when small changes are under examination to give some idea of the errors involved in the determinations concerned. Accordingly, the average variances of the results are given for each series [see Fisher, 1938, p. 53], and from these have been calculated the standard deviations of the means in each case and also the standard deviations of the differences of means. A useful measure of the significance of the results is thus obtained, though it is realized that the standard deviations themselves are probably of limited accuracy. However, application of the "t-test" [Fisher, 1938, p. 128] to the differences allows of conclusions as to significance which are in agreement with those obtained by the method shown.

Treatment of sawdust

The material available for examination was a sample of teak sawdust and, in the first series of treatments, this was used in its raw condition. The treatments applied are shown below, each treatment and the subsequent furfuraldehyde determinations being carried out in quadruplicate.

a_1. The wood (0·5–1·0 g.) was treated with 50 ml. N NaOH, and the mixture kept at room temperature for 18 hr. 50 ml. N HCl were then added, and furfuraldehyde distillation was carried out after the further necessary adjustment of HCl concentration.

a_2. A similar mixture of wood and 50 ml. N NaOH was gently boiled under reflux for 1 hr. 50 ml. N HCl were added, and furfuraldehyde distillation was then proceeded with.

a_3. The wood was boiled under reflux for 1 hr. with 100 ml. of 50% alcohol containing 25 ml. of N NaOH (i.e. 50% alcohol-1% NaOH). The alkali was then neutralized and the alcohol removed by distillation *in vacuo* at the lowest possible temperature; a further 25 ml. each of N NaOH and HCl were added before furfuraldehyde distillation.

a_4. This and the two following treatments represent additional controls for a_3. After refluxing for 1 hr. with 100 ml. of 50% alcohol, the alcohol was removed as in a_3. 50 ml. each of N alkali and acid were then added and furfuraldehyde was determined after the necessary adjustments of volume and HCl concentration.

a_5. As a_4, but the reflux treatment was with water instead of with 50% alcohol.

a_6. As a_5, again with distilled water; the reflux boiling was omitted, but the vacuum distillation necessary for volume adjustment was retained.

a_7. It will be noted that in all the above, and in b below, furfuraldehyde distillation was carried out in presence of 2·93 g. of NaCl. To indicate the influence on the furfuraldehyde yield of distillation in presence of this amount of salt, normal determinations were carried out without addition of N NaOH and N HCl.

b. In this general control, the wood was mixed with 50 ml. each of N NaOH and N HCl, and the furfuraldehyde distillation was then immediately proceeded with.

The results from these treatments are shown in Table 3, the furfuraldehyde means being calculated on the dry wood.

Table 3

Treatment	Furfuraldehyde (mean) %	$b \sim a$	$\frac{b \sim a}{0{\cdot}085}$	Furfuraldehyde yield is
b	8·09 ± 0·060	—	—	—
a_1	8·09 ± 0·060	0·00 ± 0·085	0·0	—
a_2	7·79 ± 0·060	0·30 ± 0·085	3·5	Decreased
a_3	7·82 ± 0·060	0·27 ± 0·085	3·2	Decreased
a_4	8·00 ± 0·060	0·09 ± 0·085	1·1	—
a_5	8·13 ± 0·060	0·04 ± 0·085	0·5	—
a_6	8·09 ± 0·060	0·00 ± 0·085	0·0	—
a_7	8·31 ± 0·060	0·22 ± 0·085	2·6	Increased

Average variance of results	0·014533
Average variance of mean of quadruplicates	0·003633
Standard deviation of mean of quadruplicates	± 0·060
Standard deviation of difference of means	± 0·085

It will be observed that treatments a_2 and a_3 cause significant reduction in furfuraldehyde yield; the vacuum distillation (as in a_5 and a_6) used for removal of alcohol appears to be without influence on the results of a_3. The influence of a_1 is not of itself significant, though there is a possible contribution here to the loss in a_3. Treatment a_1 has no measurable effect, nor has a_5 which involves boiling with water. The importance of carrying out the whole of the treatments a_1–a_6 and b in presence of a standard amount of NaCl is clear, since examination of the results of b and a_7 shows that there is a significant decrease in furfuraldehyde yield in presence of NaCl.

Removal of fatty material is usually recommended as a preliminary to wood analysis, and accordingly a further sample of the sawdust was exhaustively extracted successively with ether and alcohol (95 %), losing thereby 8·64 % of its dry substance. The extracted residue was then submitted to treatments a_2, a_3, a_7 and b. The results, shown in Table 4, are calculated to the original, unextracted dry wood.

Table 4

Treatment	Furfuraldehyde (mean) %	$b \sim a$	$\frac{b \sim a}{0{\cdot}096}$	Furfuraldehyde yield is
b	7·78 ± 0·068	—	—	—
a_2	7·09 ± 0·068	0·69 ± 0·096	7·2	Decreased
a_3	7·45 ± 0·068	0·33 ± 0·096	3·4	Decreased
a_7	7·80 ± 0·068	0·02 ± 0·096	0·2	—

Average variance of results	0·018558
Average variance of mean of quadruplicates	0·004639
Standard deviation of mean of quadruplicates	± 0·068
Standard deviation of difference of means	± 0·096

Treatment a_2 has again resulted in a significant decrease in the yield of furfuraldehyde, the loss in this series being apparently much greater than in the previous one. a_3, also, has again caused a decrease, but its magnitude is comparable with that of the previous series. Of great interest is the fact that the apparent depressing effect of NaCl on furfuraldehyde yield has now disappeared.

Treatment of hemicellulose

In view of the treatment losses revealed above, it is of obvious interest to know whether similar losses would be encountered with purified hemicellulose. Accordingly, a preparation of free teak hemicellulose was made by the following method. Teak sawdust was extracted with 0·5% ammonium oxalate solution in the usual way, and the residue was extracted at room temperature with successive quantities of 4% NaOH solution, each extraction occupying 2 hr. The extracts were combined, filtered through pulp and acidified with acetic acid. No attempt at fractionation was made, the hemicelluloses being precipitated together by addition of an equal volume of acetone. The washed precipitate was redissolved, and submitted to the purification treatment described below as treatment (iii). Treatments a_2, a_3, a_7 and b were carried out on the product, this time in duplicate. The results, calculated on the dry, ash-free hemicellulose, are shown in Table 5.

Table 5

Treatment	Furfuraldehyde (mean) %	$b \sim a$	$\frac{b \sim a}{0{\cdot}72}$	Furfuraldehyde yield is
b	54·23 ± 0·51	—	—	—
a_2	47·84 ± 0·51	6·39 ± 0·72	8·9	Decreased
a_3	51·25 ± 0·51	2·98 ± 0·72	4·1	Decreased
a_7	53·13 ± 0·51	1·10 ± 0·72	1·5	Decreased ?

Average variance of results	0·5176
Average variance of mean of duplicates	0·2588
Standard deviation of mean of duplicates	±0·51
Standard deviation of difference of means	±0·72

It is with some reserve that statistical analysis is applied to these results of duplicate determinations, and the influences of a_2 and a_3 are in fact so clear that such analysis seems unnecessary. However, if the results of Table 5 and the furfuraldehyde figures of Tables 6 and 7 (calculated on dry, ash-free, lignin-free hemicellulose) are combined, 9 duplicate determinations become available, the means of which, with the exception of a_2 of Table 5, occupy the very limited range of 51·25–55·40. It seems reasonable, therefore, to calculate an average variance of the mean of duplicates from these figures, and hence to determine the appropriate standard deviations. It is felt that considerable confidence can be placed in the figures so obtained; their magnitudes are shown with Table 5. It will be seen that the differences b–a_2 and b–a_3 are clearly significant, whilst that of b–a_7 is of doubtful significance.

Hemicellulose purification methods

Since the object of the alcoholic-NaOH treatment as a preliminary to hemicellulose extraction was to reduce the lignin content (or the possibility of lignin contamination) of the subsequent hemicellulose preparations, it was desired to obtain information as to the extent of such contamination when the treatment was omitted. Further, in view of the solubility of lignin in solvents such as

alcohol, the possibility exists that such solvents used in the precipitation and drying of hemicelluloses might themselves reduce the contamination to a low figure [cf. Norman, 1937, 1]. With a view to using conditions which would favour the dissolution of lignin, sawdust was extracted for 1 hr. with boiling 4% NaOH. The extract after filtration through pulp was acidified with acetic acid. No fractionation was attempted, and an equal volume of acetone was immediately added. The acetone, whilst precipitating hemicellulose, had the effect of re-dissolving the bulk of the brownish precipitate—presumably lignin—thrown down by the acid. The precipitated hemicellulose, after washing with 50% acetone, was taken to dryness by alcohol treatment and divided into three portions:

(i) Control: crude hemicellulose preparation.

(ii) This portion was redissolved in 4% NaOH, the solution neutralized with acetic acid and the hemicellulose reprecipitated with an equal volume of acetone. The product was washed with 50% acetone and thereafter taken to dryness.

(iii) After redissolving in 4% NaOH solution, an equal volume of mixed Fehling's solution was added and a volume of acetone also equal to that of the original solution. The precipitate was dissolved in 2*N* HCl and the hemicellulose reprecipitated with 1·5 vol. acetone. The precipitate was washed with (*a*) dilute HCl containing 60% of acetone and (*b*) 60% acetone. These washings are very necessary if a maximum elimination of ash is to be achieved. The product was then taken to dryness with the usual alcohol treatment.

The three products, distinguished by the above numbers, were investigated for ash, lignin content and furfuraldehyde yield. The lignin determination was made by the 72% H_2SO_4-18% HCl method of Schwalbe as used earlier [Preece, 1931]. It has been objected that the method probably tends to give results rather in excess of the true values; this, however, is of little importance in the present instance, where the error (if any) is likely to be constant in the comparative series concerned. Furfuraldehyde was determined by the normal distillation process. The results are shown in Table 6. It was also intended to carry out protein determinations on all three products. However, product (i) was found to contain not more than 0·08% N which, with the quantities available for examination, lay within the limits of experimental error. Accordingly, further protein determinations were not proceeded with.

Table 6

	Preparation no.		
	(i) %	(ii) %	(iii) %
On dry hemicellulose:			
Ash	4·74	4·06	0·22
Lignin	2·53	2·69	2·02
Furfuraldehyde	47·75	49·13	52·42
On dry, ash-free hemicellulose:			
Lignin	2·68	2·80	2·03
Furfuraldehyde	50·11	51·16	52·50
On dry, ash-free, lignin-free hemicellulose:			
Furfuraldehyde	51·49	52·63	53·58

Taking preparation (i) as the standard, the furfuraldehyde value (corrected for ash and lignin) is significantly increased by the copper treatment but not by simple re-solution and reprecipitation. The lignin figures are too few in number for reliable analysis, but it would appear unsafe to assume that any clearly

significant changes in lignin content have occurred as a result of the purification methods employed. The influence of these methods on the ash content of the products is too clear to need comment.

Alcoholic-NaOH pretreatment and hemicellulose composition

Finally, two preparations of free hemicellulose (unfractionated) were made from teak. Preparation (iv) was made by the method of Norris & Preece [1930], but omitting the alcoholic-NaOH pretreatment; preparation (v) was obtained using the full method of these authors. Both products were purified by method (iii) described in the previous section, and the results of analysis of the purified products are shown in Table 7.

Table 7

	Preparation no.	
	(iv)	(v)
	%	%
Yield, on dry wood:		
Dry, ash-free hemicellulose	1·29	1·33
On dry, ash-free hemicellulose:		
Lignin	2·28	0·81
Furfuraldehyde	53·30	54·95
On dry, ash-free, lignin-free hemicellulose:		
Furfuraldehyde	54·75	55·40

Clearly, the alcoholic-NaOH pretreatment has resulted in an important decrease in the lignin content of the preparation concerned. On the present figures, it cannot be assumed that the apparent increase in furfuraldehyde due to the pretreatment is significant. It may be noted that the lignin content of preparation (iv) is of the same order as that of each of (i), (ii) and (iii). Further, the furfuraldehyde content of the dry, ash-free preparation (iv) agrees well with the corresponding figure of a_7, Table 5. The yields of (iv) and (v) are substantially identical.

Discussion

It is apparent that treatment of plant materials with hot alkaline solutions may result in considerable modification of the furfuraldehyde-yielding substances present, leading to marked decreases in furfuraldehyde yield. A similar effect is seen with hot alkaline treatments of extracted hemicelluloses. In the present experiments with raw wood, boiling 4% NaOH and boiling 50% alcohol-1% NaOH give effects of similar magnitude, but with ether-alcohol-extracted wood and with the isolated hemicellulose mixture the influence of boiling 4% NaOH is approximately double that of the alcoholic NaOH. The actual losses with boiling 4% NaOH are, expressed as percentages of the total furfuraldehyde, 3·7, 8·9 and 11·8% for raw wood, extracted wood and isolated hemicellulose respectively. For the alcoholic NaOH treatment the corresponding figures are 3·3, 4·2 and 5·5%. It is noteworthy that the losses, particularly with the former treatment, increase markedly as the purity of the furfuraldehyde-yielding material increases. Whilst there is no direct evidence to explain this, it may well be that concomitant substances exert a protective action which is probably at least partly mechanical, since pre-extraction of wood with ether-alcohol definitely increases the effect of aqueous NaOH.

For obvious purposes of control, the principal determinations shown in Tables 3, 4 and 5 were carried out in presence of NaCl, and the variable influence of the salt is not without importance. NaCl showed a significant inhibiting effect in the raw wood and no detectable change in the extracted wood, whilst with the

pure hemicellulose it gave an increase in yield, statistically of doubtful significance. Such a result was not anticipated, it having been expected that NaCl would give a regular increase in yield throughout. It is not proposed to discuss in detail the probable reasons for the facts observed; possibly, other concomitants in addition to NaCl are capable of influencing the furfuraldehyde yield, the observed effect depending on the sum of the influences (positive or negative) of each.

A variety of circumstances has doubtless contributed to the fact that the figures for ether-alcohol-extracted wood are regularly somewhat lower than those for raw wood. Factors such as those above might play a part, as might also the solution of furfuraldehyde-yielding substances in these solvents; the latter possibility is of little importance in the case of woods. More important is the possibility of distillation with the furfuraldehyde of fatty materials, which would accordingly tend to produce an artificially high result. With teak the influence of such materials is probably unusually high. It seems desirable that, for high accuracy, this possibility should be borne in mind when carrying out the determination on natural products.

Turning now to the isolated hemicelluloses themselves, a number of significant facts emerge. Contamination with protein is of negligible proportions when mature wood is the raw material used. Small amounts of lignin pass into the preparations, and cannot be eliminated by copper treatment. It is of interest that the material determined as lignin is substantially less in amount when alcoholic-NaOH is used for pretreatment, and there is accordingly justification for the original suggestion that such pretreatment would result in enhanced purity, at least so far as lignin content is concerned. The pretreatment in the present case has neither increased nor decreased the hemicellulose yield, and the furfuraldehyde yield from the hemicellulose though apparently slightly higher is not significantly so. The degrading effect of the alkali might have been expected to cause a decrease in hemicellulose yield, but the loss due to this has been counterbalanced by increased dissolution of other material.

How far should these results be allowed to influence the choice of methods for hemicellulose preparation? Simple solution of hemicellulose material during the pretreatment is not necessarily disadvantageous if it is recognized that such extraction does take place; incidentally, no important extraction of a lignosaccharide was demonstrable in the case of teak. On the whole, it would appear that the advantages of the treatment are outweighed by its disadvantages, and it should therefore be omitted. In its place, extraction with alcohol alone might be useful in some cases for the removal of pigments, fatty materials, and the like. Alternatively, alcoholic-NaOH treatment in the cold as used by Buston [1934] may have advantages with certain materials [cf. Norman, 1937, 2, p. 57], so long as its possible solvent action for hemicelluloses is examined in each case. It must not be assumed that it is no longer necessary to investigate the relationship between lignosaccharide (or, if it be preferred, hemicelluloses soluble in hot alcoholic-NaOH) and those dissolved by cold NaOH solutions.

Clearly, cold 4 % aqueous NaOH is unobjectionable for use as a hemicellulose extractive agent when judged by the absence of degradative effect, but the position is otherwise if this solvent is used at raised temperatures. However, the difficulty already cited at the outset of this paper still persists, viz. that cold 4 % NaOH does not in all cases remove the whole of the polyuronide hemicelluloses. Until this difficulty is overcome it may be necessary, despite its disadvantages, to retain the treatment when it succeeds in extracting polyuronide material. If, in fact, it has the added disadvantage of also dissolving cellulosan, e.g. of the para-xylan type, this is to be regretted but must be accepted.

SUMMARY

1. Using teak sawdust as raw material, and from the evidence of important reductions in furfuraldehyde yield, it is shown that boiling alcoholic and aqueous NaOH solutions exert degradative effects on hemicelluloses.

2. Pretreatment with boiling alcoholic NaOH may reduce the lignin content of subsequent hemicellulose preparations, but the disadvantages of this pretreatment outweigh its advantages.

3. In view of the limited solvent action of cold 4% NaOH, it may not be possible at present to dispense with boiling extraction in all cases.

4. The influence of concomitant substances on furfuraldehyde yield from hemicelluloses is noted.

REFERENCES

Angell & Norris (1936). *Biochem. J.* **30**, 2159.
—— —— & Resch (1936). *Biochem. J.* **30**, 2146.
Buston (1934). *Biochem. J.* **28**, 1028.
Dorée (1933). Methods of Cellulose Chemistry. London.
Fisher (1938). Statistical Methods for Research Workers. Edinburgh and London.
Norman (1935). *Biochem. J.* **29**, 545.
—— (1937, 1). *Biochem. J.* **31**, 1579.
—— (1937, 2). Biochemistry of Cellulose, Polyuronides, Lignin, etc. Oxford.
Norris & Preece (1930). *Biochem. J.* **24**, 59.
Preece (1931). *Biochem. J.* **25**, 1304.

37. THE MANOMETRIC ESTIMATION OF NITRITE IN SOLUTION AND IN TISSUE

By J. BROOKS and J. PACE

From the Low Temperature Research Station, University of Cambridge and Department of Scientific and Industrial Research, and Smithfield Laboratory (Department of Scientific and Industrial Research)

(*Received 17 January 1940*)

Baumgarten & Marggraff [1930] showed that nitrites can be estimated by measuring the volume of N_2 liberated by the action of excess amidosulphonic acid since the rate of reaction (I) is rapid compared with the rate of decomposition of HNO_2 (II).

$$HNO_2 + NH_2SO_3H \rightarrow H_2SO_4 + H_2O + N_2, \quad \text{(I)}$$

$$3HNO_2 \rightarrow HNO_3 + 2NO + H_2O. \quad \text{(II)}$$

It has been found that the range and convenience of the method are increased by the use of the manometric apparatus of Van Slyke or Warburg.

Materials

Amidosulphonic acid. SO_2 was bubbled through a saturated solution of hydroxylamine hydrochloride [Raschig, 1887] for 48 hr. at room temperature and the mixture kept at $-10°$ for a further 3–4 days. The crystals of NH_2SO_3H were recrystallized from H_2O ($50° \rightarrow -10°$).

Sodium nitrite. A.R. $NaNO_2$ was twice recrystallized and analysed volumetrically by the method of Adie & Wood [1900] as modified by Cool & Yoe [1933]. The solid contained $99{\cdot}5 \pm 0{\cdot}1$ % $NaNO_2$; stock solutions were stable indefinitely. Other chemicals were of A.R. quality and contained no nitrite. Recalibrated pipettes were used for the delivery of $NaNO_2$ solutions.

Tissue. Pork muscle (loin), after rigor, was used; different samples showed no difference in behaviour. The mean water content was 76 g. H_2O/100 g. tissue.

Analysis of nitrite solutions

Warburg apparatus. The $NaNO_2$ solution was placed in the main compartment of the vessel [Warburg & Kubowitz, 1929] and the NH_2SO_3H solution[1] in the side bulb. When thermal equilibrium at 25° was reached the contents of the side bulb were tipped into the main compartment; the pressure increase reached a steady value in less than 10 min. Duplicate readings agreed to within 0·5 mm. Brodie solution, which, with the vessels used (volume about 19 ml. and v_F 1·4 ml.), corresponded to about 0·85 μl. N_2 or $2{\cdot}62 \times 10^{-6}$ g. $NaNO_2$. Table 1 gives the results obtained with 1 ml. $NaNO_2$ solution containing $6{\cdot}9 \times 10^{-4}$ g. solid/ml. and 0·4 ml. 1·7 % NH_2SO_3H solution. Only a slight excess of NH_2SO_3H (0·5–1·0 mol.) was required [Baumgarten & Marggraff, 1930] but the same results were obtained with more concentrated solutions.

[1] Hydrolysis of NH_2SO_3H in aqueous solution at room temperature is hardly detectable after several days [Baumgarten & Marggraff, 1930]. The dry solid kept over H_2SO_4 appears to be stable indefinitely.

Table 1

1 μl. $N_2 = 3{\cdot}08 \times 10^{-6}$ g. $NaNO_2$

Vessel	g. $NaNO_2$ found	% purity of $NaNO_2$
1	$6{\cdot}861 \times 10^{-4}$	99·45
4	6·874	99·63
6	6·866	99·51

The values for the percentage purity of the solid $NaNO_2$ are in good agreement with the results of the volumetric analysis and of similar accuracy. Results with more dilute solutions (taking the percentage purity as 99·5) are given in Table 2.

Table 2

1 ml. $NaNO_2$ solution: 0·4 ml. 1·7% NH_2SO_3H solution

g. $NaNO_2$ present	g. $NaNO_2$ found
$3{\cdot}42 \times 10^{-4}$	$3{\cdot}41 \times 10^{-4}$
1·37	1·37
0·68	0·69

With still more dilute solutions the percentage error becomes appreciable. The lower limit of concentration which can be analysed if an error of 3% is accepted is 10^{-5} g. $NaNO_2$/ml., using 5 ml. $NaNO_2$ solution (the maximum permissible in the available vessels). The sensitivity of the method could be increased by the use of smaller vessels (thus halving v_G and keeping v_F constant would increase the sensitivity by about 100%) or of some other form of microrespirometer, e.g. that of Heatley *et al.* [1939].

Van Slyke apparatus. In the analysis of a 2 ml. sample, one drop of octyl alcohol, 2 ml. $NaNO_2$ solution and 4·75 ml. H_2O were drawn into the gas chamber. Gas was extracted by shaking *in vacuo* for 5 min. with the Hg meniscus at the 50 ml. mark and the gas ejected. The chamber was evacuated till the solution was in the lower fourth and 0·25 ml. saturated NH_2SO_3H solution was added through a Hg seal. The contents of the chamber were shaken with the Hg meniscus at the 50 ml. mark for 5 min. and any traces of CO_2 absorbed in the usual way by the addition of 1 ml. air-free *N* NaOH. The gas was brought to a volume of 0·5 ml. (or 2·0 ml.) and the pressure p_1 read. The gas was ejected and the fluid meniscus lowered to the 0·5 ml. (or 2·0 ml.) mark and the pressure p_2 read. Then

vol. N_2/100 ml. $= f(p_1 - p_2 - c)$ and 22·4 vol. N_2/100 ml. $= 6{\cdot}9 \times 10^{-4}$ g. $NaNO_2$/ml.

f is a factor tabulated by Peters & Van Slyke [1932, p. 325]; c is a small correction obtained from a blank analysis using 2 ml. H_2O in place of 2 ml. $NaNO_2$ solution, where $c = p_1' - p_2'$.

The means of duplicate analyses are given in Table 3; the meaning of the symbols S and a is defined by Peters & Van Slyke [1932, p. 282].

Table 3

2 ml. $NaNO_2$ solution analysed; $S = 7{\cdot}0$ ml.

a ml.	Vol. N_2/100 ml. found	g. $NaNO_2$/ml. found	g. $NaNO_2$/ml. present
2·0	22·30	$6{\cdot}87 \times 10^{-4}$	$6{\cdot}86 \times 10^{-4}$
2·0	11·10	3·42	3·43
0·5	4·45	1·37	1·37
0·5	2·24	$6{\cdot}90 \times 10^{-5}$	$6{\cdot}86 \times 10^{-5}$

Duplicate analyses of 2 ml. samples agreed to within 0·03 vol./100 ml. corresponding to about 2×10^{-6} g. $NaNO_2$; the sensitivity therefore is similar to that of the Warburg apparatus. With a sample of 6 ml., $S = 7$ ml. and $a = 0{\cdot}5$ ml. a concentration of 2×10^{-5} g. $NaNO_2$/ml. can be analysed with an error of about 1 %. It was found possible, however, to analyse a much larger sample, e.g. 25 ml. The procedure was similar to that described above except that one drop of octyl alcohol, 25 ml. $NaNO_2$ solution and 2 ml. H_2O were drawn into the gas chamber and gas was extracted and ejected three times (the period of shaking in each case was 5 min. with the Hg meniscus at the 50 ml. mark). Table 4 gives calculated values of f for $S = 27{\cdot}25$ ml. and $a = 0{\cdot}5$ ml. [cf. Peters & Van Slyke, p. 282] and the results of three analyses. Duplicate analyses agreed to within 0·002 vol./100 ml. corresponding to $1{\cdot}5 \times 10^{-6}$ g. $NaNO_2$. With a 25 ml. sample therefore a concentration of 10^{-6} g. $NaNO_2$/ml. can be estimated to within 3 %.

Table 4

Sample = 25 ml.
$S = 27{\cdot}25$ ml.
$a = 0{\cdot}5$ ml.

°C.	15	16	17	18	19	20
f	0·00254	0·00253	0·00252	0·00251	0·00250	0·00249

Vol. N_2/100 ml.	g. $NaNO_2$/ml. found	g. $NaNO_2$/ml. present
0·178	$5{\cdot}48 \times 10^{-6}$	$5{\cdot}49 \times 10^{-6}$
0·0886	2·73	2·75
0·0365	1·12	1·10

The specificity of the reaction

In agreement with Lutzenko [1937] it was found that the quantitative evolution of N_2 was not affected by the presence of NaCl, $NaNO_3$, glucose, sucrose and tissue extractives. Analyses of $NaNO_2$ solutions containing these substances, using mainly the Warburg apparatus, are given in Table 5.

Table 5

1 ml. solution contained $6{\cdot}86 \times 10^{-4}$ g. $NaNO_2$

	NaCl				$NaNO_3$	NaCl and $NaNO_3$		Glucose	Sucrose	Tissue extractives*
g./100 ml.	3	6	10·8	25	4·5	20	4·5	5	5	
g. $NaNO_2$/ml. $\times 10^4$ found	6·90	6·86	6·86	6·90	6·85	6·83		6·86	6·79	6·80

* 50 g. minced tissue were stirred with 50 ml. 10·8 % NaCl solution for 2 hr. and the filtrate added to an equal volume of $NaNO_2$ solution.

When there is a marked difference between the concentrations of the solutions in the two compartments of the Warburg vessel a vapour pressure correction (obtained from a blank experiment with the solutions in the absence of $NaNO_2$) must be added to or subtracted from the observed pressure increase.[1] With the

[1] The pressure of H_2O vapour in the vessel is mainly determined by the vapour pressure of the solution in the main compartment. When the latter pressure is altered by the addition of NH_2SO_3H solution the pressure increase is the sum of the change in vapour pressure in the vessel and the change due to the evolution of N_2. With concentrated salt solutions in the main compartment the magnitude of the correction can be decreased by increasing the concentration of the solution in the side bulb, e.g. by using 0·4 ml. saturated NH_2SO_3H solution.

dilute solutions of Tables 1 and 2 the correction was negligible ($<0{\cdot}2$ mm. Brodie solution) but became appreciable with the solutions of Table 5. For the analysis of solutions of the latter type it is better to use the Van Slyke apparatus as the correction c is less dependent on the composition of the sample.

When a solution contained NO_2^- and HCO_3^- or $CO_3^=$, N_2 and CO_2 were liberated by NH_2SO_3H. In the Van Slyke apparatus CO_2 was absorbed by the routine addition of 1 ml. air-free NaOH and in the Warburg apparatus by the usual method of placing 0·3 ml. 5% KOH solution and a small roll of filter paper in the central tower of the vessel.

It should be noted that the estimation of nitrite contained in strongly buffered solutions may require the addition of increased amounts of NH_2SO_3H since a low pH is required for the reaction.

The estimation of nitrite in tissue

A known amount of $NaNO_2$ was mixed with tissue and the mixture analysed; this usual test of an analytical method was complicated by reaction between nitrite and tissue constituents.

(A) *Van Slyke apparatus.* Nitrite was estimated by the analysis of an aqueous extract of the tissue (without preliminary removal of dissolved protein). Samples of 10 g. minced tissue were thoroughly mixed with 1 ml. of $NaNO_2$ solution (containing $6{\cdot}86 \times 10^{-3}$ g. $NaNO_2$) and the mixtures, after storage at 25° for various periods, were transferred to 500 ml. flasks. In each case the volume of tissue and washings was made up to 500 ml. with H_2O and the mixture gently shaken for 1 hr. After filtration, 25 ml. filtrate were analysed in the Van Slyke apparatus. In some cases 1 ml. $NaNO_2$ solution was added to 10 g. tissue previously mixed thoroughly with 1 g. solid NaCl. The mean of duplicate analyses is given in Fig. 1, the times give the interval between addition of $NaNO_2$ to the tissue and dilution with H_2O.

Of the added $NaNO_2$ 97% was recovered after 30 min. contact with tissue. There was a slow disappearance of nitrite; the rate was increased by about 70% by the presence of 1 g. NaCl/10 g. tissue. Extrapolation of the linear plots in Fig. 1 to zero time gave values of 98 and 99% recovery. The method therefore is reasonably accurate; the small initial deficit appears to be due mainly (*v. infra*) to a rapid reaction of nitrite with tissue constituents. Since a concentration of 10^{-6} g. $NaNO_2$/ml. filtrate can be estimated to within 3% an initial concentration of about 50×10^{-6} g. $NaNO_2$/g. tissue can be estimated with the same accuracy. The amount of tissue extracted could however be increased, thus using 50 g. tissue the limiting concentration giving an error of 3% would be 10^{-5} g. $NaNO_2$/g. tissue.

(B) *Warburg apparatus.* With certain limitations nitrite in tissue can be estimated by the action of NH_2SO_3H on the tissue. Pork muscle, after rigor, has a small residual respiration but the R.Q. is sufficiently near unity to give no pressure change in the Warburg vessel during a determination.[1]

1 g. minced or thinly-sliced tissue was spread on the bottom of the main compartment and covered with 1 ml. $NaNO_2$ solution.[2] After 30 min. contact

[1] The O_2 uptake (in this case using KOH in the central tower) was found to be about 0·5 μl./g./min. at 25°. After the addition of NH_2SO_3H the uptake was about 0·2 μl./g./min.

[2] This procedure was equivalent to but more convenient than using 1 g. tissue containing added $NaNO_2$ and 1 ml. H_2O. Experiments showed that in 30 min. the uniform distribution of $NaNO_2$ between the solution and the H_2O of the muscle was at least 95% complete.

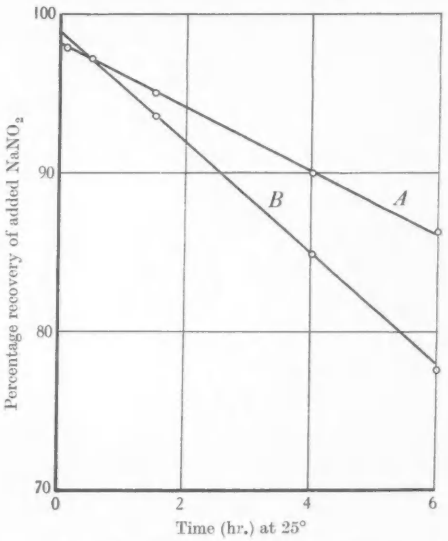


Fig. 1. Percentage recovery of $NaNO_2$ added to tissue (*A*) and to tissue containing 10% NaCl (*B*). The percentage recovery is given by $100\,(500 - 2{\cdot}4)$ (g. $NaNO_2$/ml. filtrate)/$6{\cdot}86 \times 10^{-3}$, where 2·4 ml. are taken as the volume of the non-aqueous portion of 10 g. tissue.

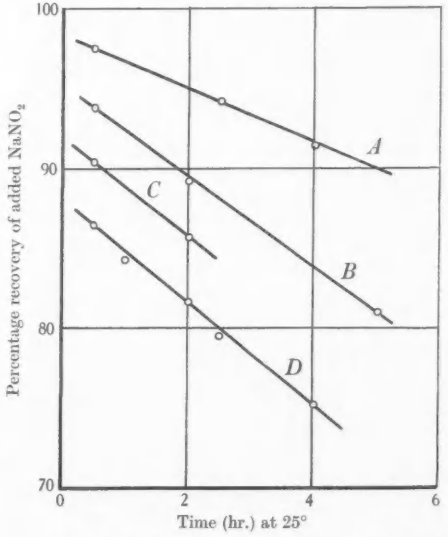


Fig. 2. Percentage recovery of $NaNO_2$ from 1 g. tissue (*A*) and from 1 g. tissue in the presence of 3×10^{-2} g. NaCl (*B*), 6×10^{-2} g. NaCl (*C*) and $10{\cdot}8 \times 10^{-2}$ g. NaCl (*D*).

0·4 ml. saturated NH_2SO_3H solution[1] was added from the side bulb. At the *p*H of pork muscle the amount of bicarbonate present should be negligible; blank experiments in the absence of $NaNO_2$ gave a pressure decrease of 1 mm. Brodie solution (taken as the vapour pressure correction). The density of the tissue was taken as 1·05 in the calculation of v_F and v_G.

Ten experiments using 1 ml. solution containing $6{\cdot}86 \times 10^{-4}$ g. $NaNO_2$ gave a mean value of 97·5 % recovery. Experiments with longer periods of contact before the addition of NH_2SO_3H (Fig. 2, curve *A*) showed that there was a linear rate of disappearance of nitrite of a similar magnitude (about 2 % per hour) to that previously observed. Extrapolation of the curve to zero time gave a value of 98·5 % recovery.

Table 6 gives the results obtained using different amounts of $NaNO_2$; the time of contact of tissue and $NaNO_2$ was 30 min. The values for the percentage recovery are not constant; this appears to be mainly due, not to experimental error, but to a rapid disappearance of nitrite of the order of 10^{-5} g. $NaNO_2$/g. tissue (Table 6, column 4).

Table 6

Side bulb—0·4 ml. saturated NH_2SO_3H solution
Main compartment—1 g. tissue and 1 ml. $NaNO_2$ solution

g. $NaNO_2$ added	g. $NaNO_2$ found	% recovery	g. $NaNO_2$ lost
$6{\cdot}86 \times 10^{-4}$	$6{\cdot}69 \times 10^{-4}$	97·5	$1{\cdot}7 \times 10^{-5}$
3·43	3·33	97	1·0
1·37	1·30	95	0·7
0·69	0·62	90	0·7

The results obtained by methods A and B are in good agreement. Method B is more convenient but less sensitive than method A since the weight of tissue in the Warburg vessel could not be increased above 2 g.

The action of NH_2SO_3H on tissue containing added NaCl

The action of NH_2SO_3H on tissue containing $NaNO_2$ and high concentrations of NaCl gave unexpected results and nitrite could not be estimated by the addition of NH_2SO_3H to such tissue. In these experiments 1 g. minced or sliced tissue was placed in the Warburg vessel together with 1 ml. solution containing $6{\cdot}86 \times 10^{-4}$ g. $NaNO_2$ and various amounts of NaCl. The side bulb contained 0·4 ml. saturated NH_2SO_3H solution (the same results were obtained using 0·75 ml.). Vapour pressure corrections were obtained from blank experiments in the absence of $NaNO_2$.

It was found (Fig. 2) that (*a*) the percentage recovery decreased with the time of contact of tissue and $NaNO_2$, (*b*) the rate of disappearance of nitrite was increased by the presence of NaCl, (*c*) extrapolation of the linear plots to

[1] With 0·4 ml. NH_2SO_3H solution of different concentrations in the side bulb the following values were obtained. The use of a larger volume of saturated NH_2SO_3H solution did not increase further the percentage recovery. The lower values obtained with the more dilute solutions of NH_2SO_3H may be due to the buffering power of the tissue:

% NH_2SO_3H	3·0	5·0	Saturated
% recovery	84·7	94·0	97·3

As would be expected the time required (30–45 min.) for the attainment of a steady pressure increase after the addition of NH_2SO_3H was greater than that observed when NH_2SO_3H was added to a nitrite solution.

zero time showed an initial deficit which was proportional to the amount of NaCl present. Table 7 gives the extrapolated values of percentage recovery at zero time.

Table 7

1 g. tissue plus 1 ml. solution

g. NaCl/ml. (*a*)	% recovery at zero time	$-\Delta$ (% recovery) (*b*)	*b*/*a*
0	98·5	—	—
3×10^{-2}	95·5	3·0	1·0
6	92·0	6·5	1·1
10·8	88·1	11·4	1·0

Further experiments showed that (*a*) the initial deficit was proportional to the concentration of NaCl and not to the amount, (*b*) the effect was reversible with respect to the NaCl concentration; thus the addition of H_2O to a tissue-NaCl-$NaNO_2$-H_2O mixture after a given time increased the percentage recovery obtained on adding NH_2SO_3H to a value corresponding to the lowered concentration of NaCl. The initial deficit therefore was not due to destruction of nitrite; this was confirmed by almost complete extraction of $NaNO_2$ with excess H_2O (99 % for zero time of contact) from tissue containing 10 % NaCl (Fig. 1). Further, since NaCl and tissue extractives had no effect on the quantitative evolution of N_2 in the absence of tissue (Table 5) it seems probable that part of the $NaNO_2$ present in a tissue structure containing relatively high concentrations of NaCl is not accessible to the action of NH_2SO_3H.

Discussion

The manometric estimation of nitrite in solution is a convenient alternative to the usual volumetric methods [cf. Cool & Yoe, 1933] especially when the solution contains substances which interfere with volumetric analysis. Although small amounts of nitrite can be estimated manometrically the range of the method described is smaller than that of colorimetric analysis;[1] the procedure of the Association of Official Agricultural Chemists [1935] allows the estimation of as little as $0{\cdot}3 \times 10^{-6}$ g. $NaNO_2$.

Nitrite in tissue is usually estimated colorimetrically after extraction with H_2O and removal of dissolved protein. The procedure of Lewis & Blake [1932, p. 433], applied to mixtures of tissue and nitrite and to samples of bacon gave results which agreed to within 7 % (the error of the colorimetric method is from 5 to 10 %) with those obtained by the manometric technique. In these analyses the $NaNO_2$ content was greater than 50×10^{-6} g./g. tissue; the colorimetric method can be used to estimate concentrations of the order of 10^{-6} g. $NaNO_2$/g. tissue.

The results indicate that nitrite reacts rapidly with tissue constituents in amounts of the order of 10^{-5} g. $NaNO_2$/g. tissue; reaction with haemoglobin and myoglobin in the muscle would probably account for a large part of this initial nitrite loss. There is also a slow disappearance of nitrite in contact with

[1] Numerous modifications of the Griess-Ilosvay colour reaction have been suggested. In some cases the reagents, sulphanilic acid and α-naphthylamine, are used in acetic acid solution. It was found when the nitrite solution contained NaCl that the depth of colour developed depended on the concentration of NaCl. When HCl was substituted for acetic acid, as in the methods of the A.O.A.C. [1935] and Lewis & Blake [1932, p. 420], the presence of up to 25 % NaCl in the nitrite solution had no effect on the estimation.

tissue but the cause is unknown. Using the Warburg apparatus, a small gas absorption was detectable over long periods of contact of nitrite with tissue (< 1 mm. Brodie solution/hr.) but the amount was too small to account for the disappearance on the basis of atmospheric oxidation of $NaNO_2$ catalysed by a tissue enzyme.

Summary

1. Manometric methods for the estimation of nitrite are described. The methods are based on the reaction of NH_2SO_3H with nitrite in the Van Slyke or Warburg apparatus.

2. The quantitative evolution of N_2 from nitrite and NH_2SO_3H is not affected by the presence of NaCl, $NaNO_3$, sucrose, glucose and muscle extractives.

3. Nitrite in muscle can be estimated by the manometric analysis of a H_2O extract of the tissue. With certain limitations nitrite in muscle can be estimated directly by the action of NH_2SO_3H on tissue contained in the Warburg apparatus.

4. On mixing muscle and $NaNO_2$ there is both a rapid disappearance of a small amount of nitrite (of the order of 10^{-5} g. $NaNO_2$/g. tissue) and a slow disappearance of nitrite.

The work described above was carried out as part of the programme of the Food Investigation Board, and is published by permission of the Department of Scientific and Industrial Research.

REFERENCES

Adie & Wood (1900). *J. chem. Soc.* **77**, 1076.

Association of Official Agricultural Chemists (1935). Methods of Analysis, p. 506. Washington D.C.: A.O.A.C.

Baumgarten & Marggraff (1930). *Ber. dtsch. chem. Ges.* **63**, 1019.

Cool & Yoe (1933). *Industr. Engng Chem.* (Anal. Ed.), **5**, 112.

Heatley, Berenblum & Chain (1939). *Biochem. J.* **33**, 53.

Lewis & Blake (1932). Allen's Commercial Organic Analysis, 5th ed. **9**, 420, 433. London: Churchill.

Lutzenko (1937). *Zh. prikl. Khim., Leningr.*, **10**, 948.

Peters & Van Slyke (1932). Quantitative Clinical Chemistry, **2**, 282, 325. London: Baillière, Tindall and Cox.

Raschig (1887). *Ber. dtsch. chem. Ges.* **20**, 584, 1158.

Warburg & Kubowitz (1929). *Biochem. Z.* **214**, 5.

38. NATURAL ANTHOCYANIN PIGMENTS

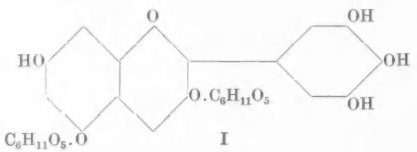
3.[1] FLOWER PIGMENTS OF *VERBENA HYBRIDA*

BY ROSE SCOTT-MONCRIEFF (MRS O. MEARES)
AND VIOLET CELIA STURGESS

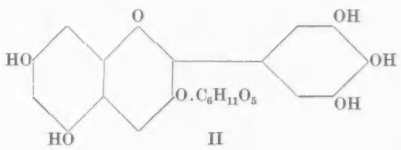
John Innes Horticultural Institution, Merton

(*Received 26 January 1940*)

IN connexion with genetical studies of flower colour in *Verbena hybrida* [Beale, 1940] we have isolated the anthocyanins from flowers of two colour types, purple and maroon. The pigment of the purple is a delphinidin 3:5-diglycoside (I) and of the maroon a delphinidin 3-monoglycoside (II); the sugars are probably glucose:



I



II

Both pigments on acid hydrolysis gave a hexahydroxyflavylium chloride which proved to be delphinidin chloride. That obtained from purple *Verbena* was fused with caustic potash; the products were phloroglucinol and gallic acid.

Delphinidin 3:5-diglucoside (delphin) has been isolated from the bright blue flowers of *Salvia patens* by Reynolds *et al.* [1934]; it has also been synthesized by the same workers. The colours of the pure natural and synthetic products, in a range of buffer solutions from *p*H 3·2 to 14·0, were identical. The pigments of *S. patens* and purple *Verbena* similarly showed no significant divergence. This does not however establish the identity of their sugars. The anthocyanins most frequent in nature are glucosides, and since we obtained glucosazone from the sugars produced by acid hydrolysis of the purple *Verbena* pigment, this is probably delphinidin 3:5-diglucoside.

Delphinidin-3-glucoside has also been synthesized [Reynolds & Robinson, 1934], but apparently it has not yet been isolated in the pure condition from natural sources. However, the occurrence of a delphinidin 3-monoside mixed with other pigments has been noted in several plants, including *Viola tricolor* and *Vaccinium Myrtillus*. A delphinidin 3-monoside has also been isolated from Awobana paper by Kuroda [1933]. The pigment of the maroon *Verbena* gave the same colours over a range of *p*H as those described for synthetic delphinidin 3-β-glucoside by Reynolds & Robinson [1934]. The distribution number of the natural pigment to *iso*amyl alcohol was a little higher than that of the synthetic product, but this is probably not significant, as Reynolds & Robinson found a similar variation between the natural and synthetic 3:5-diglucosides with *n*-butyl alcohol.

[1] Part 1 (1930) *Biochem. J.* **24**, 753; Part 2 (1930) *Biochem. J.* **24**, 767.

Experimental

A. *Isolation of pigment from purple* Verbena *flowers*

The fresh flowers were extracted with 1 % aqueous HCl, and the solution shaken repeatedly with ethyl acetate to remove flavones and tannins. After several extractions with ethyl acetate, solid anthocyanin began to separate from the acid solution. This increased in amount as the flavone was removed. When the extractions were completed the solution was allowed to settle overnight. The liquid was then decanted and the remaining sludge centrifuged. The solid was collected and boiled with 0·5 % aqueous HCl, which dissolved the pigment and left behind some white insoluble material which was discarded. On cooling the filtered solution, golden-brown crystals of semi-pure anthocyanin separated. This was further purified by dissolving in hot 0·5 % aqueous HCl, cooling and making the acid concentration up to 5 %. The pigment obtained by this method was clean but amorphous. Two or three crystallizations from 0·5 % aqueous HCl gave the pure anthocyanin in lens-shaped leaflets, blue by transmitted light and with a gold reflex.

The anthocyanin was not extractable from an aqueous acid solution by *iso*amyl alcohol, and it showed all the reactions of a delphinidin 3:5-dimonoside; for example it gave a pure blue colour with Na_2CO_3, violet with $FeCl_3$ in neutral solution, and with NaOH blue which faded rapidly. The addition of a large volume of hot water to an alcoholic solution of the pigment transformed it to the colourless pseudo-base, from which the coloured form was restored by acidification. (Found, after drying at 105° over P_2O_5 *in vacuo*: C, 48·8; H, 4·8; Cl, 3·9 %. $C_{27}H_{30}O_{17}$, 0·75 HCl, 0·25 H_2O requires C, 49·2; H, 4·75; Cl, 4·04 %.)

There is a loss of HCl on drying at 105° in a vacuum. This was also noticed [Reynolds *et al.* 1934] when the pigment of *Salvia patens* was dried under similar conditions. In this case there is partial replacement of HCl by water.

The colours in buffered solutions of graded *p*H were compared with those of a pure specimen of delphin from *S. patens*. There were no marked divergences, but the purple *Verbena* pigment faded rather more rapidly between *p*H 3·4 and 5·0, and the solutions were slightly redder than those of the *Salvia* pigment between *p*H 3·8 and 8·6. The differences were too slight to be of any structural significance.

Preparation of anthocyanidin. The diglycoside was hydrolysed by boiling with 16 % aqueous HCl for 3 min. The anthocyanidin separated as a dark brown amorphous precipitate, which was dissolved in the minimum quantity of cold 0·5 % aqueous HCl, heated to boiling and the acid concentration increased to 10 %. On cooling, the anthocyanidin separated in clusters of rod-shaped crystals. These were filtered, dried *in vacuo* at 105° over P_2O_5 and analysed. (Found: C, 52·3; H, 4·1; Cl, 6·9 %. $C_{15}H_{10}O_7$, 2/3 HCl, 1·0 H_2O requires C, 52·3; H, 3·7; Cl, 6·8 %.)

The anthocyanidin gave all the qualitative reactions of delphinidin chloride. It was extracted by amyl alcohol from 1 % aqueous HCl. The amyl alcohol on shaking with sodium acetate became violet blue, and on addition of a drop of $FeCl_3$ solution this changed to pure blue. The pigment was not extracted by either the "cyanidin reagent" or the "delphinidin reagent" [Robinson & Robinson, 1931]. The comparison of this anthocyanidin with that from the maroon *Verbena* and with synthetic delphinidin chloride are described below (p. 271).

The amounts of sugar and anthocyanidin produced on acid hydrolysis were estimated as follows. A weighed amount of anthocyanin was hydrolysed, cooled

and left overnight to ensure as complete a precipitation of the anthocyanidin as possible. It was then filtered, the residue washed with a small quantity of 16 % aqueous HCl and dried to constant weight over P_2O_5 at 105°. The filtrate was shaken with amyl alcohol to remove the last traces of anthocyanidin, then with ether to remove amyl alcohol, and finally neutralized with caustic soda. The sugars were then estimated by Bertrand's method. In the first hydrolysis 30·2 mg. of the air-dried delphinidin 3:5-dimonoside (28·0 mg. calculated as anhydrous) gave 13·8 mg. anthocyanidin (49·3 %) and 11·11 mg. hexose, calculated as glucose (40·0 %). In a second estimation 42·6 mg. of the diglycoside (dried *in vacuo* over P_2O_5 at 105°) gave 20·6 mg. anthocyanidin (48·3 %) and 18·53 mg. glucose (43·5 %). $C_{27}H_{31}O_{17}Cl$ requires $C_{15}H_{11}O_7Cl$, 51·1 %; $C_6H_{12}O_6$, 54·2 %. These results are similar to those obtained by Reynolds *et al.* who found values for sugar 9–12 % below the theoretical expectation.

In a larger scale experiment 0·395 g. of anthocyanin was hydrolysed and the sugar converted into its osazone in the usual way. This was crystallized from pyridine—ethyl alcohol—water mixture and identified as glucosazone by its M.P., 205–206°, which was not depressed by admixture with an authentic specimen.

Fusion with potash. 0·4 g. of air-dried anthocyanidin chloride was heated for 2 min. at 200° with 5 g. solid KOH and 5 ml. water. The mixture was cooled, diluted with water, acidified and extracted several times with ether. The acidic and phenolic degradation products were separated with bicarbonate in the usual manner and found to be phloroglucinol and gallic acid. The former was identified by its transient violet colour with $FeCl_3$, red colour with a pine shaving and conc. HCl, the formation of an orange-red diazobenzene compound, and by the brick-red phloroglucide of methylfurfuraldehyde obtained by heating rhamnose with 20 % HCl and distilling the vapour into a solution of the phenol in water. The gallic acid gave a blackish-blue precipitate with $FeCl_3$, a deep blue colour with $FeSO_4$, and its alkaline solution darkened rapidly on exposure to air.

B. *Isolation of pigment from maroon* Verbena *flowers*

The fresh flowers were extracted with 1 % aqueous HCl, and the solution shaken with successive amounts of *n*-butyl alcohol; about 5 l. of the alcohol were used in all. The combined butyl alcoholic extracts were shaken with an equal volume of light petroleum (B.P. 60–80°) and 500 ml. of 1 % aqueous HCl. The concentrated aqueous solution so obtained was extracted several times with ethyl acetate in order to remove flavones. 250 ml. absolute alcohol and 2 l. of ether were then added, when the pigment was precipitated as a dark red syrup. The ether-alcohol was decanted, the syrup warmed gently on the water bath to remove any remaining ether and left for 16 days. It then contained dark red crystals of the pigment together with some colourless crystalline material. The crystals were filtered and boiled with 1 % aqueous HCl, which dissolved the pigment but not the white substance, the latter being removed by filtration of the hot solution. The filtrate was shaken with benzene to remove traces of alcohol, an equal quantity of conc. HCl added and the solution left for several days, when clean amorphous pigment separated. This was dissolved in the minimum quantity of hot 0·5 % aqueous HCl, the solution filtered, cooled and 0·1 vol. 25 % ethyl alcoholic HCl added. Only slight separation had taken place after 2 days, and the bulk of the pigment was recovered by the addition of $\frac{1}{3}$ vol. of conc. HCl. Crystallization was effected by solution in warm methyl alcohol and addition of an equal volume of 10 % methyl alcoholic HCl. Irregular masses of crystals separated, bluish-purple by transmitted light with a copper

reflex. These were dried at 105° *in vacuo* over P_2O_5, and analysed. (Found: C, 48·86; H, 4·5; Cl, 7·2 %. $C_{21}H_{21}O_{12}Cl, H_2O$ requires C, 48·6; H, 4·44; Cl, 6·85 %.)

The distribution number (*iso*amyl alcohol and equilibrated 0·5 % aqueous HCl) was determined by the standard method [Levy & Robinson, 1931] with the following results:

		Distribution no.
I. 5·0 mg./100 ml. mixed solvents:	1st shaking	12·6
	2nd shaking	13·8
II. 4·0 mg./50 ml. mixed solvents:	1st shaking	11·4
	2nd shaking	12·1

The anthocyanin is therefore monoglycosidic. Its colour reactions in solutions of graded *p*H [Robertson & Robinson, 1929] were examined, and found to be the same as those of synthetic 3-β-glucosidyldelphinidin chloride [Reynolds & Robinson, 1934]. The pigment was only slightly soluble in hot ethyl alcoholic HCl. It gave a blue colour with Na_2CO_3, violet with $FeCl_3$ in neutral solution, blue violet with sodium acetate, and a pure blue, which faded rapidly, with 10 % NaOH.

Preparation of anthocyanidin. 23·4 mg. of anthocyanin (dried *in vacuo* at 105° over P_2O_5) were dissolved in 0·5 % aqueous HCl, and hydrolysed by boiling for 3 min. with an equal volume of conc. HCl. The anthocyanidin chloride separated in clusters of crystals on cooling. These crystals were air-dried, weighed and analysed. (Found: C, 49·4; H, 4·2; Cl, 9·5 %. $C_{15}H_{11}O_7Cl$, 1·5 H_2O requires C, 49·2; H, 3·8; Cl, 9·7 %.)

Willstätter & Weil [1916] described four hydrates of delphinidin chloride, dependent upon the condition of crystallization. Among them was one with 1·5 H_2O crystallizing from 20 % HCl.

The sugars produced in the above hydrolysis were determined by Bertrand's method. The results were as follows: hexose calculated as glucose, 5·887 mg., 25·2 %; anthocyanidin, 17·13 mg., air-dried, 73·2 % (calculated as anhydrous). $C_{21}H_{21}O_{12}Cl$ requires $C_6H_{12}O_6$, 36 %; $C_{15}H_{11}O_7Cl$, 67·6 %.

A 1 % aqueous HCl solution of the anthocyanidin gave the same qualitative reactions as those of delphinidin chloride from the purple *Verbena* (see above, p. 269).

The colours of the anthocyanidin chlorides from the purple and maroon *Verbena* were compared with those of a specimen of synthetic delphinidin chloride. The colours were identical except from *p*H 8·6 to 9·2, at which points the anthocyanidin from the purple type was slightly more violet than the other two.

Miss V. C. Sturgess desires to acknowledge a grant from the Department of Scientific and Industrial Research.

REFERENCES

Beale (1940). *J. Genet.* (in the Press).
Kuroda (1933). *Proc. imp. Acad. Japan*, **9**, 94.
Levy & Robinson (1931). *J. chem. Soc.* p. 2715.
Reynolds & Robinson (1934). *J. chem. Soc.* p. 1039.
—— —— & Scott-Moncrieff (1934). *J. chem. Soc.* p. 1235.
Robertson & Robinson (1929). *Biochem. J.* **23**, 35.
Robinson & Robinson (1931). *Biochem. J.* **25**, 1687.
Willstätter & Weil (1916). *Liebigs Ann.* **412**, 178.

39. STUDIES IN DETOXICATION

5. THE PREPARATION OF *d*-GLUCURONE FROM AMMONIUM MENTHYLGLUCURONATE

By R. TECWYN WILLIAMS

From the Physiology Department, Medical School, Hospitals Centre, Birmingham

(*Received 29 January 1940*)

SINCE *d*-glucuronic acid is an essential constituent of many biologically important products (e.g. heparin, certain immuno-polysaccharides and sex hormone glucuronides), its preparation is of the utmost importance. It is, however, still difficult to obtain synthetically on a large scale. Synthetic methods have been devised by Fischer & Piloty [1891], Smolenski [1923], Zervas & Sessler [1933] and recently by Stacey [1939]. These methods are difficult to carry out and, apart from the last, the yields are poor. Yields of 5% of glucuronic acid were obtained by Weinmann [1929] from gum arabic, whilst Kalb & Falkenhausen [1927] prepared it in small yield by oxidation of cellulose.

The best methods for obtaining glucurone (the form in which glucuronic acid is usually isolated) are those depending upon the glucuronic acid detoxication mechanism of mammals. The old method for its large-scale preparation [Thierfelder, 1887; Fischer & Piloty, 1891] was from the conjugated glucuronide, euxanthic acid [cf. Robertson & Waters, 1931] which, as the magnesium salt, is the main constituent of Indian yellow or puree, a pigment obtained from the urine of cows fed on mango leaves. This pigment, however, is no longer available in large quantities. Quick's method [1927], using zinc bornylglucuronate, gives good yields, but after much experience with it [e.g. Pryde & Williams, 1933] I find the method described here to be more convenient [cf. Williams, 1939, 1] and to give much larger yields of glucurone. A method of preparing glucuronic acid from ammonium *l*-menthylglucuronate has been described by Ehrlich & Rehorst [1925].

The success of the biosynthetic method depends on (*a*) the choice of a suitable glucuronogenic drug which conjugates to a large extent in a conveniently managed animal, (*b*) an easy method of isolating the conjugated product in good yield from the urine and (*c*) a suitable method for isolating glucurone from the glucuronide. The method described below appears to satisfy these criteria. Nearly 60% of the glucuronogenic drug (*dl*-menthol) used is excreted by the rabbit conjugated with glucuronic acid [Williams, 1938] and nearly all of the conjugated compound can be isolated from the urine as the ammonium salt. The crude dry ammonium salt contains 85% ammonium menthylglucuronate, 10% ammonium sulphate and 5% water; this salt should therefore yield, on hydrolysing, 42–43% of its weight as glucurone ($C_6H_8O_6$) and 38% as menthol. In actual practice, using the method described below, about 80% of the theoretical amount of glucurone can be recovered on the 50–100 g. scale. The overall yield of crude glucurone is therefore 39–40 g. for every 100 g. of *dl*-menthol fed.

Experimental

Preparation of ammonium menthylglucuronate. Rabbits (2–2·5 kg.) were given 3 g. of *dl*-menthol as a partial emulsion in warm water by stomach tube. The tube was washed down with warm water so that the total water given was about 50 ml. The rabbits were then put in cages placed in tinned-copper funnels and the urine collected for about 30–40 hr. The urine and funnel washings were filtered through muslin and made alkaline with a few drops of ammonia (sp. gr. 0·880), then raised to the boil and quickly filtered[1] to remove precipitated phosphates. Ammonium sulphate (50 g./100 ml. urine) was gradually added to the filtrate with occasional shaking, the solution being simultaneously heated to boiling. The hot solution was immediately filtered[1] to remove a small precipitate of protein[2] and the filtrate kept overnight at 0°, whereby ammonium menthylglucuronate crystallized and filled the solution. The salt was sucked dry, pressed to remove the last traces of mother liquor and then dried *in vacuo* over $CaCl_2$. The yield of dry crude salt was 1·4 g./g. of *dl*-menthol fed. This preparation has been successfully carried out some 30 times during the past 3 years. Table 1 gives the results of five experiments carried out recently. The same yield was obtained from rabbits which had no previous feeding with menthol (or any other compound) as from rabbits which at some time or other had been fed with menthol.

Table 1. *Preparation of ammonium menthylglucuronate*

Exp. no.	No. of rabbits used	Total *dl*-menthol fed* g.	Time of urine collection hr.	Yield dry NH_4 salt g.	Yield per g. menthol fed g.	$[\alpha]_D$ of 2% aqueous solution
1	8	24	43	34·0	1·42	−27·7°
2	3	9	43	13·4	1·49	−28·3°
3	1	3	19	3·8	1·27	−28·6°
4	8	24	43	32·8	1·37	−28·3°
5	4	12	43	17·1	1·42	−26·3°

* Dose 3 g./rabbit.

The salt dissolved in water to give a clear colourless solution and specimen 1 (see Table 1) contained 4·7 % H_2O. Specimens 1 and 2 were analysed for sulphate and contained 6·7 and 7·55 % SO_4 respectively or 9·2 and 10·4 % calc. as $(NH_4)_2SO_4$; neither specimen gave any ash when calcined. The specific rotations quoted in Table 1 show that all five specimens have the same composition. This salt therefore contains about 85 % anhydrous ammonium menthylglucuronate ($C_{16}H_{27}O_7NH_4$) and should yield 43·4 % of glucurone ($C_6H_8O_6$).

Preparation of glucurone. A typical preparation was carried out as follows: 50 g. of the above salt were shaken with 400–500 ml. water. Most of the salt dissolved and its solution was complete on adding 100 ml. of 2*N* H_2SO_4. The free menthylglucuronic acid was extracted from the solution with six 400 ml. portions of ether and after the sixth extraction the residual aqueous solution gave only a faint napthoresorcinol reaction. It was not necessary to dry the ethereal extract, in fact other experiments showed that drying, especially with

[1] For quick filtration, Green's 450 grey crepe filter papers were found to be very satisfactory.

[2] It was stated in Part III [Williams, 1939, 2] that this protein was probably a mucoid. It is, however, mainly denatured albumin. Rabbits receiving a 3 g. dose of *l*- or *dl*-menthol, *d*- or *dl-iso*menthol, *l*-menthone or *d-neo*menthol show a slight albuminuria. Tests on control rabbit urine showed no albuminuria.

LINDA HALL LIBRARY
KANSAS CITY, MO.

anhydr. Na_2SO_4 resulted in a loss of glucuronide owing to considerable adsorption of the latter on the sulphate. The adsorbed glucuronide could not be re-extracted with ether.

The ethereal solution was evaporated under slightly diminished pressure in a 500 ml. distilling flask fitted with a continuous feed arrangement. The residue was a coloured syrup which crystallized completely on cooling. The menthylglucuronide was now dissolved in 200 ml. 0·4 *N* H_2SO_4 with heating and the solution simultaneously hydrolysed and steam-distilled until no more menthol distilled. In this experiment the menthol was recovered in two fractions thus:

(1) 4·4 g. showing $[\alpha]_D^{21°} + 28·5°$ ($c = 2$, in alcohol),
(2) 13·5 g. showing $[\alpha]_D^{21°} + 8·3°$ ($c = 2$, in alcohol)

[cf. Williams, 1939, 2]. About 18 g. of menthol were recovered from a possible 19·2 g.

The solution after removal of menthol was treated with a slight excess of "Analar" lead acetate solution (16 g. in 100 ml. water) [cf. Stacey, 1939] and the precipitated lead sulphate was removed by centrifuging. The clear centrifugate and washings were treated with H_2S to precipitate any lead present, as sulphide which was then filtered off under suction. Colloidal PbS was removed by adding a little blood charcoal and filtering. After aeration to eliminate H_2S, the pale yellow clear solution (*ca.* 500 ml.) was concentrated to a thin syrup at 50° *in vacuo*. This syrup deposited 6·6 g. of almost pure glucurone, M.P. 176–177°, on standing at room temperature overnight. These crystals were collected, washed with methyl alcohol and the filtrate again concentrated *in vacuo* to a syrup. The syrup was dissolved in methyl alcohol and the solution deposited 5·6 g. of glucurone. Concentration to a syrup and treatment with methyl alcohol was thrice repeated and the following amounts of glucurone obtained, 2·3, 1·8 and 2·2 g. The last fraction was coloured, but in the first four were white (rotations are recorded in Table 2). A total yield of about 18 g. was therefore obtained from a possible 21·5 g.; 16 g. were obtained in a fairly pure state. This yield corresponds to 39–40 g. glucurone for every 100 g. *dl*-menthol fed. It was further purified by recrystallization from methyl alcohol.

Table 2. *Preparation of glucurone*

Fraction	Yield g.		M.P.*	$[\alpha]_D^{19°}$ in 1% aqueous solution*
1	6·6		176–177°	+20·7°
2	5·6			
3	2·3	mixed	157°	+19·7°
4	1·8			
5	2·2		132°	+21·0°

* Stacey [1939] gives M.P. 176–178° and $[\alpha]_D$ +20°.

A strong aqueous solution of glucurone was added to a solution of 2:4-dinitrophenylhydrazine in acid (H_2SO_4) alcohol. On standing at room temperature for 30 min. nodular masses of bright yellow micro-needles, which appeared to be the 2:4-*dinitrophenylhydrazide* of *glucuronic acid*, separated. The compound was washed with water containing HCl and then with water. It crystallized from hot water as long deep yellow needles, M.P. 205° (decomp.), and from hot alcohol as masses of bright yellow micro-needles, M.P. and mixed M.P. with the deep yellow form, 205° (decomp.). It reduced Fehling's solution on boiling and

dissolved in dilute NaOH giving a deep purplish-red solution which was decolored on acidifying. (Found: C, 38·7; H, 4·0; N, 15·0 %; $C_{12}H_{14}N_4O_{10}$ requires C, 38·5; H, 3·8; N, 15·0 %.)

SUMMARY

A new method is described for the preparation of glucurone from ammonium menthylglucuronate isolated from the urine of rabbits fed with *dl*-menthol. A yield of about 39–40 g. of glucurone per 100 g. *dl*-menthol fed was obtained.

REFERENCES

Erhlich & Rehorst (1925). *Ber. dtsch. chem. Ges.* **58**, 1989.
Fischer & Piloty (1891). *Ber. dtsch. chem. Ges.* **24**, 521.
Kalb & Falkenhausen (1927). *Ber. dtsch. chem. Ges.* **60**, 2514.
Pryde & Williams (1933). *Biochem. J.* **27**, 1197, 1205.
Quick (1927). *J. biol. chem.* **74**, 331.
Robertson & Waters (1931). *J. chem. Soc.* p. 1709.
Smolenski (1923). *Roczn. Chem.* **3**, 153.
Stacey (1939). *J. chem. Soc.* p. 1529.
Thierfelder (1887). *Hoppe-Seyl. Z.* **11**, 388.
Weinmann (1929). *Ber. dtsch. chem. Ges.* **62**, 1637.
Williams (1938). *Biochem. J.* **32**, 1849.
—— (1939, 1). *Nature, Lond.*, **143**, 641.
—— (1939, 2). *Biochem. J.* **33**, 1519.
Zervas & Sessler (1933). *Ber. dtsch. chem. Ges.* **66**, 1326.

40. EXCRETION OF ANDROGENS BY EUNUCHS: THE ISOLATION OF 17-KETOSTEROIDS FROM THE URINE

BY NANCY HELEN CALLOW AND
ROBERT KENNETH CALLOW

From the National Institute for Medical Research, London, N.W. 3

(*Received 1 February 1940*)

THE result of investigations in the last few years has been to show that the excretion in human urine of material with androgenic action is an imperfect index of the androgenic activity of the gonads. In spite of some degree of correlation of androgen excretion with sex and age, it would be impossible in practice to determine from the androgen content of the urine either the sex or the age of a human individual. Even after castration, although reports have been made to the contrary, it seems that the excretion of androgens still occurs, and may be within normal limits [Callow *et al.* 1940]. Very low levels of androgen excretion may be associated with castration, Addison's disease or severe pituitary insufficiency. A high level, on the other hand, is the only observation with a definite and independent diagnostic value; it occurs in cases of adrenal cortical tumour.

In a series of papers from this laboratory, efforts have been made to apply chemical methods to the analysis of the androgenic material in extracts of human urine, with the object of replacing the unspecific biological test. It was found that the androgenic material in extracts of hydrolysed urine was concentrated in the ketonic fraction, confirming Butenandt & Tscherning [1934], and that the biological activity was actually parallel to the content of the 17-ketosteroids determined colorimetrically [Callow *et al.* 1938]. The attempt to separate individual compounds from the ketonic fraction led to the isolation of androsterone, aetiocholan-3(α)-ol-17-one and *trans*dehydroandrosterone from men's and women's urine in similar amounts [Callow & Callow, 1939]. It had been found that, of these substances, androsterone and aetiocholan-3(α)-ol-17-one could probably be derived from testosterone given by injection [Callow, 1939], although it seemed improbable that in women they were derived from secreted testosterone, whilst *trans*dehydroandrosterone was found in large amount in the urine of cases of adrenal cortical tumour [Crooke & Callow, 1939]. In a recent paper [Callow & Callow, 1939], we expressed the hope that chemical investigation of the nature of the compounds excreted by eunuchs and ovariectomized women would give a clue to the contribution of the adrenal cortex to the urinary 17-ketosteroids. Hirschmann [1939] has now isolated androsterone, aetiocholan-3(α)-ol-17-one and *trans*dehydroandrosterone from the urine of ovariectomized women by methods similar to ours, and in yields only slightly below those obtained from normal women's urine. Hansen [1938] first attempted to identify the androgens in eunuchs' urine, but could only conclude, from the rather uncertain evidence of type of biological activity, that they resembled those of normal men's urine but differed from androsterone.

We made a preliminary examination of a small quantity of mixed extracts from the urine of eunuchs and found that it yielded androsterone, aetiocholan-3(α)-ol-17-one and *trans*dehydroandrosterone, though in quantities insufficient for certain identification. In the investigation described in the present paper, we examined a large amount of urine from an individual eunuch and confirmed the presence in it of these three compounds which occur in bulked collections of normal urine. The only difference from the normal shown by the eunuch's urine was an increase in the total amount of *trans*dehydroandrosterone and in its proportion to the other two 17-ketosteroids. It is, therefore, clear that androsterone and aetiocholan-3(α)-ol-17-one are not of purely gonadal origin in either sex, whether derived from testosterone or from some other compound, and they may be transformation products of substances from the adrenal cortex.

The individual eunuch whose urine we examined may not be a typical case, since the level of his excretion of 17-ketosteroids was the highest among the eunuchs we examined, but the results give support to the supposition that *trans*dehydroandrosterone is of adrenal cortical origin, since the relative amount of this compound excreted appears to be increased when the alternative source of urinary 17-ketosteroids is absent. Further, this observation is consistent with the hypothesis that increased activity of the adrenal cortex may, in some cases, compensate for the absence of gonads. This is analogous to the hypothesis put forward by Hamblen *et al.* [1939], that the adrenal cortex compensates by increased activity for the regression of gonadal activity at the onset of the climacteric in women.

Experimental

Source of urine

The eunuch, E. C., aged 20, was a patient of Dr A. W. Spence at St Bartholomew's Hospital. A brief case history has been published [Case 5, Callow *et al.* 1939, 1]. He had been treated with testosterone propionate, but when collection of urine for the present investigation was begun he had received no recent treatment, and a 24 hr. collection showed a daily excretion of 9·4 mg. of 17-ketosteroids, as compared with 10·9 mg./day a year previously, before any hormone therapy; the oestrin excreted was also lower, according to biological assays, for which we are indebted to Dr C. W. Emmens. Subsequent collections were made discontinuously and sent to the laboratory at fortnightly intervals; the specimens were fairly concentrated (average sp. gr. 1·021). Toluene was used as a preservative, and the specimens on receipt had *p*H 5–6. We are very grateful to Dr A. W. Spence for the opportunity of obtaining this material, and to the subject himself, who very patiently continued with the collections over a period of 6 months.

Extraction of ketonic material

The separate collections of urine were extracted as soon as received by the combined hydrolysis and extraction method [Callow *et al.* 1939, 2], and the neutral fractions from about 24 l. of urine, with colorimetric values for 17-ketosteroid content ranging from 10·0 to 15·4 mg./l., were combined and treated with the Girard-Sandulesco reagent P. From 1·35 g. there were obtained 0·296 g. of ketonic and 0·706 of non-ketonic material.

Separation by adsorption and elution

The ketonic fraction was dissolved in carbon tetrachloride ("Analar") and the solution put through a chromatograph column, 20 × 1 cm., prepared with carbon tetrachloride and alumina (Merck's "Aluminium oxide standardized

according to Brockmann"). The column was developed first with carbon tetrachloride and then with carbon tetrachloride containing 0·1 % ethanol. The weights of fractions obtained after the collection and evaporation of successive portions of eluate are shown in Table 1. Further elution with carbon tetrachloride containing 0·2 % ethanol yielded only traces of gummy material in a concentration of 0·01 mg./ml.

Table 1

Fraction	Vol. of eluate ml.	Solvent	Wt. of fraction mg.	Conc. mg./ml.	Remarks
I	300	CCl_4	12·5	0·042	Crystalline. Chloro-compound?
II	275	CCl_4	13·7	0·05	Gum
III	500	CCl_4	2·8	0·0056	Gum
IV	220	0·1 % EtOH in CCl_4	19·3	0·086	Gum, containing *trans*-dehydroandrosterone
V	230	0·1 % EtOH in CCl_4	52·8	0·23	Crystalline *trans*dehydroandrosterone
VI	220	0·1 % EtOH in CCl_4	15·7	0·071	Crystalline. Androsterone
VII	190	0·1 % EtOH in CCl_4	7·0	0·037	Gum
VIII	570	0·1 % EtOH in CCl_4	22·7	0·0398	Red gum, yielded aetiocholan-3(α)-ol-17-one
IX	280	0·1 % EtOH in CCl_4	5·3	0·019	Crystalline

Isolation of steroids from crude fractions

(*a*) trans*Dehydroandrosterone.* Fraction IV was treated in 90 % alcohol with digitonin. The precipitate, after drying and decomposition of the pyridine solution with ether, yielded 5·7 mg. of material, which was benzoylated. The benzoate, after recrystallization from ethyl acetate, melted at 233–245°. This was presumably *trans*dehydroandrosterone benzoate, but the amount was too small for further purification and confirmation of its identity. The main bulk of the *trans*dehydroandrosterone was contained in the following fraction. Fraction V had $[\alpha]_D + 36°$, $[\alpha]_{5461} + 47°$ in EtOH. It was converted into the benzoate by treatment with benzoyl chloride in pyridine. The crude, crystalline benzoate (60 mg.) obtained by diluting with water, yielded, with two successive crystallizations from ethyl acetate, fine needles having M.P. 250–254° (24 mg.) and finally with M.P. 253–254° (16 mg.), unchanged when mixed with an authentic specimen of *trans*dehydroandrosterone benzoate. Transition of the needle form to a platy form at 236–239° was observed. The total amount of *trans*dehydroandrosterone present in Fractions IV and V is estimated to be about 40–50 mg. or about 2 mg./l.

(*b*) *Androsterone.* The crystalline material from Fraction VI melted at 140–170° but a portion which sublimed on the cover-slip melted at 178–182°. The whole fraction had $[\alpha]_D + 74°$ and $[\alpha]_{5461} + 81°$ in EtOH. After successive recrystallizations from methanol and from ethyl acetate material was obtained with M.P. 184–185°. Mixed with an authentic specimen of androsterone (M.P. 184–185°) it had M.P. 183·5–184·5°. By evaporation of the mother liquors from the above recrystallization crystals were obtained which were rapidly washed with a little ether to remove gummy, yellow material, and then treated with benzoyl chloride in pyridine. The reaction mixture was diluted with water, extracted with ether, and the extract washed, dried and evaporated. The residue, after two recrystallizations from methanol, yielded needles, M.P. 172–

175°, unchanged when mixed with an authentic specimen of androsterone benzoate. A rough estimate of the amount of androsterone present, assuming that some also came in Fraction V, gives the figure of 0·5 mg./l. of original urine.

(*c*) *Aetiocholan-3(α)-ol-17-one.* Fraction VIII was treated with charcoal in methanol solution to remove the red colour, filtered and the filtrate evaporated to dryness. The residue, scratched after addition of a trace of methanol, yielded crystals, M.P. 136–145°, $[\alpha]_D$ +92°, $[\alpha]_{5461}$ +115° in EtOH. After two recrystallizations from methanol the M.P. was 146–147·5° (soft at 145°). A mixture with an authentic specimen of aetiocholan-3(α)-ol-17-one (M.P. 151–152°), for which we are indebted to Prof. Ruzicka, melted at 147–151°. A benzoate was prepared from the mother liquors of the aetiocholan-3(α)-ol-17-one, which, after three recrystallizations from methanol, had M.P. 161–163·5° alone, and 161–164° when mixed with an authentic specimen of aetiocholan-3(α)-ol-17-one benzoate (M.P. 161·5–163·5°). The amount of aetiocholan-3(α)-ol-17-one present in the original urine is roughly estimated at 0·8–0·9 mg./l.

(*d*) *Unidentified material.* Fraction I was partly crystalline. Treated with methanol, a small fraction of it was sparingly soluble. The filtrate from this deposited crystals, M.P. 138–155°, which contained about 90 % of 17-ketosteroid, determined colorimetrically. It showed only faint selective absorption in the ultraviolet in alcoholic solution. It contained halogen (Cu wire test). In view of the small quantity it was not examined further. Possibly this fraction consists of chlorinated compounds such as the 3-chloroandrosten-17-one obtained by Butenandt & Dannenbaum [1934]. Similar fractions have been obtained from extracts of normal urine.

Note. All melting points recorded were observed in Kofler's micro-melting point apparatus. The optical rotations were measured in a 4 dm. tube.

Summary

Examination of the urine of a eunuch has been made by the same methods as those which have been applied to normal urines. From the ketonic fraction there were isolated the same compounds as are obtained from normal men's or women's urine, namely, androsterone, aeticholan-3(α)-ol-17-one and *trans*dehydroandrosterone.

The relative amounts of the compounds in a eunuch's urine, about 0·5, 0·9 and 2 mg./l., respectively, compared with those in mixed normal men's urine (1·6, 1·4 and 0·2 mg./l., respectively) show a probably significant increase in the amount of *trans*dehydroandrosterone.

None of these compounds is of purely gonadal origin. The results are consistent with the assumption that *trans*dehydroandrosterone is derived from the adrenal cortex, which may be more active in a eunuch than in a normal subject.

REFERENCES

Butenandt & Dannenbaum (1934). *Hoppe-Seyl. Z.* **229**, 192.
—— & Tscherning (1934). *Hoppe-Seyl. Z.* **229**, 157.
Callow, N. H. (1939). *Biochem. J.* **33**, 559.
Callow, N. H. & Callow, R. K. (1939). *Biochem. J.* **33**, 931.
—— —— & Emmens (1938). *Biochem. J.* **32**, 1312.
—— —— —— (1939, 1). *J. Endocrinol.* **1**, 99.
—— —— —— (1940). *J. Endocrinol.* (in the Press).
—— —— —— & Stroud (1939, 2). *J. Endocrinol.* **1**, 76.
Crooke & Callow, R. K. (1939). *Quart. J. Med.* **32**, 233.
Hamblen, Ross, Cuyler, Baptist & Ashley (1939). *Endocrinology*, **25**, 491.
Hansen (1938). *Endokrinologie*, **21**, 9.
Hirschmann (1939). *J. biol. Chem.* **130**, 421.

41. STUDIES IN TISSUE METABOLISM

12. THE ACTION OF COLCHICINE ON TRANSPLANTED, INDUCED AND SPONTANEOUS MOUSE TUMOURS

By ERIC BOYLAND and MARGARET ESTHER BOYLAND[1]

From The Chester Beatty Research Institute, Royal Cancer Hospital (Free), London, S.W. 3

(*Received 1 February 1940*)

In a previous paper [Boyland & Boyland, 1937] it was shown that spontaneous mouse tumours were very much less susceptible than grafted tumours (S 37, Mal. and Crocker-180) to the action of colchicine. Intraperitoneal injection of 0·05 mg. of colchicine into mice bearing grafted tumours caused (1) haemorrhage in the tumours, (2) reduction of the ascorbic acid content and (3) lowered tissue respiration and glycolysis. The combination of these three phenomena is called the "colchicine effect". The effects of injection of colchicine on primary induced tumours and on a grafted sarcoma, M.C.D.B. 1, which has always been grown in the same pure line of Dilute Brown mice [see Boyland & Boyland, 1939], as well as further results with spontaneous mammary tumours are described in this paper.

The grafted sarcoma, M.C.D.B. 1, has been extremely valuable in experiments on chemotherapy and in studying the effects of treatment on tumour metabolism. An analogous mammary carcinoma (C.C_3H 1) has been established in the C_3H strain of mice and its reaction to colchicine has been studied.

Experimental

Tumours used. (*a*) The M.C.D.B. 1 sarcoma which, having only grown in the same line, might be expected to bear more resemblance to a primary tumour in its behaviour than the usual strains of grafted tumours which have not been grown entirely in one line of mice.

(*b*) C.C_3H.1 refers to transplants of a carcinoma that arose spontaneously in a mouse of the C_3H strain; the tumour has been grown only in C_3H mice. The first and second grafted generations were used.

(*c*) Induced tumours were obtained by subcutaneous injections of 2 doses each of 1 mg. of methylcholanthrene in lard into mixed stock mice. They were in all cases sarcomata.

(*d*) Spontaneous mammary tumours.

Mice were injected intraperitoneally with solutions of colchicine 18–20 hr. before examination of the tumours. Respiration and anaerobic glycolysis were measured by the Warburg technique at 37°. The ascorbic acid content was determined by grinding the healthy tumour tissue with 3 volumes of 5% trichloroacetic acid and titrating the extract with 2:6-dichlorophenol-indophenol. The degree of haemorrhage was estimated macroscopically as in the previous paper.

[1] Finney-Howell Fellow.

Table 1. *Effect of injection of colchicine on the properties of tumours*

A. Dilute Brown mice bearing M.C.D.B. 1

mg. colchicine	Degree of haemorrhage in tumours	Metabolism of tumours $-Q_{O_2}$	Metabolism of tumours $Q_L^{N_2}$	Ascorbic acid content of tumours mg./g.
Control (untreated)	–	9·2	27·7	0·12
,,	–	9·4	28·8	0·10
,,	–	11·8	25·2	0·14
,,	–	9·1	34·2	0·11
,,	–	10·0	35·7	0·13
,,	–	11·4	31·0	0·12
	Mean	10·1	30·4	0·12
0·025	+	6·8	13·9	0·05
0·025	+	10·1	30·0	0·08
0·025	+ +	3·4	7·8	0·09
0·025	+ +	2·0	20·1	0·07
0·025	–	11·3	22·9	0·09
0·025	–	6·9	23·5	0·10
0·025	–	6·9	21·6	0·09
0·025	–	11·6	22·7	0·15
0·025	–	8·2	23·6	0·08
0·05	+ + +	1·9	8·0	0·06
0·05	–	7·5	22·2	0·09
0·05	+ + +	2·0	2·6	0·06
0·05	+ +	6·5	11·6	0·04
0·05	+ +	0·6	2·5	0·05
0·05	+ + +	0·6	1·6	0·03
0·05	+ +	1·5	8·2	0·04

B. C_3H mice bearing $C.C_3H.1$

mg. colchicine	Degree of haemorrhage in tumours	Metabolism of tumours $-Q_{O_2}$	Metabolism of tumours $Q_L^{N_2}$	Ascorbic acid content of tumours mg./g.
Control (untreated)	–	10·4	27·6	0·41
,,	–	14·0	23·2	0·30
,,	–	10·2	19·0	0·62
,,	–	11·0	25·5	0·30
,,	–	14·7	26·0	0·38
	Mean	12·1	24·3	0·40
0·05	–	11·2	21·2	0·08
0·05	–	11·1	23·8	0·26
0·05	–	7·2	5·0	0·29
0·05	–	12·6	15·0	.
0·05	–	14·1	29·4	.
0·05	–	12·8	27·1	0·43
0·1	+	7·0	20·0	0·06
0·1	–	4·0	28·0	0·08
0·1	+ +	0·6	3·3	0·14
0·1	–	1·2	13·3	0·16
0·1	–	4·2	11·9	.
0·1	–	10·5	8·4	.
0·1	–	0	6·4	.
0·2	+ +	0·4	2·8	0·18
0·2	+ +	0	4·6	0·16
0·2	+ +	0	2·0	0·20
0·2	+ + +	0·1	4·5	0·07
0·2	+	1·2	4·4	0·07

C. Mice bearing tumours induced with methylcholanthrene

Control (untreated)	–	6·6	23·2	0·12
,,	–	9·3	14·0	0·18
,,	–	10·7	16·5	0·13
,,	–	8·5	17·5	0·17
,,	–	10·2	18·5	0·17
	Mean	9·1	17·9	0·15
0·1	+ +	4·7	13·0	0·05
0·1	–	13·9	21·5	0·21
0·1	–	7·0	9·0	0·11
0·1	+ +	6·4	13·0	0·04
0·2	+ +	1·1	6·0	0·03
0·2	+ +	5·6	9·5	0·04
0·2	+ + +	0·5	0·6	0·05
0·2	–	5·7	12·7	0·08

D. Mice bearing spontaneous tumours*

Control (untreated)	.	12·8	25·6	0·62
,,	.	8·7	11·4	0·35
,,	.	5·3	17·0	0·16
,,	.	6·8	10·2	0·22
0·1	.	10·9	29·0	0·17
0·1	.	10·1	17·5	0·27
0·1	.	6·5	8·9	0·11
0·1	.	11·5	20·0	0·23
0·2	.	4·2	7·6	0·01
0·2	.	4·8	5·4	0·19
0·2	.	6·8	9·5	0·25
0·2	.	7·6	19·2	0·20
0·2	.	4·4	7·8	0·19
0·2	.	10·6	11·4	0·29

* Some of these results are taken from Boyland & Boyland [1937] for comparison.

Results

(*a*) Table 1A shows that 0·05 mg. in most cases produces the typical colchicine effect in M.C.D.B. 1 tumours, but this dose was often fatal; 0·025 mg. sometimes has a slight effect on the tumours but is generally ineffective; in about 20 % of cases this dose was fatal; 0·1 mg. was always fatal to these mice. It was shown [Boyland & Boyland, 1937] that all mice bearing Crocker sarcoma 180 survived a dose of 0·025 mg. and 8 out of 20 survived 0·1 mg. Thus the Dilute Brown mice with tumours are more sensitive to colchicine than stock mice with the Crocker tumour. This may be due to the relatively small size of the former mice. This transplanted tumour therefore resembles other grafted tumours previously investigated, rather than induced or spontaneous tumours, in its reaction to colchicine.

(*b*) The untreated carcinoma C.C_3H.1 is sometimes haemorrhagic, so that the estimation of the haemorrhage due to colchicine is not so definite as with the other sarcomata which have been used; however the signs + + and + + + are used where there was extensive haemorrhage caused by the treatment. Table 1B shows that 0·05 mg. colchicine sometimes had a slight effect, 0·1 mg. was generally effective and 0·2 mg. always so. The mortality with 0·2 mg. was low, 1 out of 6 mice dying within 24 hr. This tumour (in C_3H mice) is therefore more resistant to the action of colchicine than the Crocker sarcoma in mixed stock mice or the M.C.D.B. 1 sarcoma in Dilute Brown mice. It more nearly resembles induced sarcomas in mixed stock mice.

(*c*) Most of the induced tumours examined (Table 1C) were unaffected by 0·1 mg. but 0·2 mg. frequently produced the "colchicine effect".

(*d*) It is harder to determine exactly the reaction of spontaneous tumours, as there is great variation between untreated tumours, but there is no doubt

Table 2. *The effect of 2-methylnaphthoquinone and ascorbic acid with colchicine on Crocker sarcoma 180*

	Degree of haemorrhage in tumours	Metabolism of tumour		Ascorbic acid content of tumours mg./g.
		$-Q_{O_2}$	$Q_L^{N_2}$	
A. Controls (untreated)	–	7·8	22·5	0·22
	–	8·2	20·6	0·26
	–	.	.	0·22
	–	.	.	0·14
B. 0·1 mg. colchicine	+ + +	0·3	0·7	0·11
	+ +	1·0	5·3	0·22
	+ +	.	.	0·13
	+	.	.	0·08
	+	.	.	0·20
C. 50 mg. ascorbic acid	–	.	.	0·24
	–	.	.	0·21
D. 50 mg. ascorbic acid followed by 0·1 mg. colchicine	+ +	.	.	0·05
	+ +	.	.	0·17
	+	.	.	0·10
E. 0·1 mg. colchicine preceded by 3 doses of 0·01 mg. 2-methyl-naphthoquinone + 5 mg. ascorbic acid	–	6·2	20·5	0·18
	+	0·2	1·5	0·10
	+ +	0·4	0·5	0·06
	+ + +	.	.	0·09
	+ +	.	.	0·08
0·1 mg. colchicine preceded by 2 doses of 0·01 mg. 2-methyl-naphthoquinone + 40 mg. ascorbic acid	+	.	.	0·07
	+ +	0·1	1·7	0·10
	+ +	.	.	0·09

that they are much less affected than grafted tumours, and somewhat less than induced tumours, which occupy an intermediate position.

Ascorbic acid. Andervont & Shimkin [1939] showed that ascorbic acid neutralized the effect of bacterial filtrates on tumours, but we have found no such action with ascorbic acid and colchicine. Mice bearing tumours were injected with 20–50 mg. of ascorbic acid (approximately neutralized with $NaHCO_3$) a few hours before injecting colchicine. The typical colchicine effect was always observed. Some results are given in Table 2. It was shown [Boyland & Boyland, 1937] that colchicine and colchiceine reduced the respiration of normal tumour slices when added *in vitro*. This effect was not reversed if ascorbic acid (0·5 mg./ml.) was added to the incubation medium.

Vitamin K. As the mechanism of the haemorrhage production by colchicine is unknown, the effects of ascorbic acid and vitamin K were tested. Mice bearing the Crocker sarcoma were dosed daily with a suspension of 5–40 mg. ascorbic acid suspended in a cod-liver oil solution containing 0·01 mg. 2-methylnaphthoquinone which has been shown [Almquist & Klose, 1939] to have a high vitamin K activity. After 2 or 3 doses the mice were injected with 0·1 mg. colchicine and the tumours examined after 18 hr. The results are given in Table 2. It will be seen that the methylnaphthoquinone and ascorbic acid had no effect in reducing the "colchicine effect". In one mouse treated with the vitamins colchicine did not produce the typical results, but occasionally mice treated with colchicine alone escape the effects, so this cannot be considered significant.

Discussion

The effect of injecting large doses of colchicine into mice with grafted tumours is similar to the effect of bacterial filtrates. The bacterial preparations which produce haemorrhage in grafted tumours also elicit the Schwarzman phenomenon in the rabbit skin. It seemed possible that the haemorrhage produced in grafted tumours was similar to that of the Schwarzman reaction and that the tumour was already sensitized because it was a foreign or heterologous tissue. Spontaneous tumours, which are not foreign tissues, do not react to bacterial filtrates or to colchicine. Andervont [1936] found that *Bact. coli* filtrates produced haemorrhage in induced tumours and we show that colchicine has similar effects. The fact that induced tumours which must be homologous tissue do react suggests that the mechanism of the haemorrhage is different from the Schwarzman phenomenon. The strong reactions given by the M.C.D.B. 1 tumour which must also be homologous support this suggestion. Andervont & Shimkin [1939] have shown that ascorbic acid reduces the reaction of tumours to bacterial filtrates, and we find that it has no such effect with colchicine, which would suggest that the effects produced are not identical.

Summary

A spontaneous mammary carcinoma has been grafted into mice of the strain (C_3H) in which the tumour appeared, in order to obtain a grafted carcinoma of the same genetic constitution as the host.

Mice bearing (1) spontaneous mammary tumours, (2) primary tumours induced by injection of methylcholanthrene and (3) two strains of grafted tumours grown in pure lines of mice were treated with colchicine. The reactivity to colchicine increased from (1) to (3). Treatment with 2-methylnaphthoquinone and ascorbic acid does not alter the effect produced by colchicine.

We are indebted to the British Empire Cancer Campaign for their generous support. We should like to express our thanks to Messrs Glaxo Ltd. for a gift of 2-methylnaphthoquinone.

REFERENCES

Almquist & Klose (1939). *J. Amer. chem. Soc.* **61**, 1923.
Andervont (1936). *Amer. J. Cancer*, **27**, 77.
—— & Shimkin (1939). *Amer. J. Cancer*, **36**, 451.
Boyland & Boyland (1937). *Biochem. J.* **31**, 454.
—— —— (1939). *Biochem. J.* **33**, 618.

42. THE GLUTAMIC ACID OF NORMAL AND MALIGNANT TISSUE PROTEINS

By A. C. CHIBNALL, M. W. REES, E. F. WILLIAMS and E. BOYLAND

From the Biochemical Department, Imperial College, London, S.W. 7, and the Chester Beatty Research Institute, Royal Cancer Hospital (Free), London, S.W. 3

(*Received 30 January 1940*)

Kögl & Erxleben [1939, 1] have recently put forward the suggestion that partial racemization is a characteristic of the proteins of malignant tissues. The claim is based on their observation that a normal tissue such as calf lung, when submitted to a short acid hydrolysis, gave a series of amino-acids, all of which, after isolation, showed under standard conditions the normal rotation, whereas a few of the corresponding products from malignant tissues were found to be partially racemized. This difference was particularly striking in the case of glutamic acid. From normal lung and normal heart tissues they obtained, in what appeared to be good yields, the usual *l*-(+)glutamic acid showing, in 9% hydrochloric acid, the normal rotation of $[\alpha]_D + 31{\cdot}6°$. Corresponding preparations from an ovarian tumour, from an ovarian carcinoma and from two different mammary carcinomata, on the contrary, showed rotations of $[\alpha]_D + 21{\cdot}8°$, $+4{\cdot}6°$, $+11{\cdot}6°$ and $+16{\cdot}1°$, indicating that they contained 15·6, 42·7, 31·7 and 24·6% respectively of *d*-(−)glutamic acid.

The importance of these findings needs no emphasis, for they demonstrate a chemical differentiation of malignant and normal tissues which readily suggests several new paths of research into the origin and control of cancer. Confirmation in other laboratories was thus clearly desirable and as an extensive research had been in progress for some time at the Imperial College on the dicarboxylic acid content of various standard proteins the opportunity was taken to investigate the position with regard to glutamic acid.

The method of analysis which we employed in the first instance to isolate this acid as its hydrochloride was different from that of Kögl & Erxleben, and was essentially that of Foreman [1914], in which the aspartic and glutamic acids are first separated from the other amino-acid products of hydrolysis by precipitation as their calcium salts in the presence of alcohol. Applied to protein preparations from three tumours, viz. a Crocker sarcoma, a carcinoma of the bronchus and an extension in the neck from a carcinoma of bronchiole, we readily obtained in each case a first and large crop of glutamic acid hydrochloride which, on recrystallization from hydrochloric acid, had the normal rotation. The yields, calculated as glutamic acid in terms of weight of tissue preparation taken for analysis, varied between 5 and 7%, and as no attempt had been made to work up the mother liquors we knew from experience that these yields must be well below the maximum obtainable. Even so they compared favourably with all except two of those quoted by Kögl & Erxleben,[1] and we were accordingly led

[1] Kögl & Erxleben [1939, 1, 3] give many tables of tissue amino-acid analysis, in most of which appears the weight of glutamic acid obtained. It is clear however from the accompanying text that the weights quoted are really those of the hydrochlorides which they actually isolated, so that when referring to their yields of the free acid in the present paper it has been necessary for us to make the appropriate correction.

to suggest, in our preliminary communication [Chibnall *et al.* 1939], the need of a more extended investigation into these workers' claim.

Kögl & Erxleben [1939, 2, 3] have replied to this criticism, briefly in a letter to *Nature* and more extensively, with many new experimental data, in two papers (one with Akkerman [1939]), manuscript copies of which were very kindly sent to us before publication. *Inter alia* they produced evidence purporting to show that, under the modified Foreman conditions of analysis that we employed, the calcium salt of racemic glutamic acid has a much greater solubility than that of the natural antipode; furthermore they showed that the racemic hydrochloride has about twice the solubility of the corresponding natural product in either concentrated or 20% hydrochloric acid at 0°, observations which led them to doubt whether our analytical procedure was suitable for demonstrating the presence of any partially racemized acid in our hydrolysates as our, admittedly large, crop of hydrochloride obtained by treatment of the calcium precipitate had been recrystallized from a relatively large volume of hydrochloric acid before the specific rotation had been determined. Indeed certain *ad hoc* experiments, in which samples of the racemic acid had been added to a hydrolysate of calf embryo tissue before analysis by the Foreman method provided them with what they regarded as conclusive evidence in support of their supposition, for the hydrochloride finally isolated had a rotation of $[\alpha]_D + 31{\cdot}4°$ characteristic of the natural *l*-(+)glutamic acid. Finally, they reported further investigations of the partially racemized acid which they had previously obtained from a Brown-Pearce tumour and an ovarian carcinoma, leading in both cases to the isolation in pure form of the hydrochloride of the *d*-(−)glutamic acid showing, in 9% hydrochloric acid, a rotation of $[\alpha]_D - 31{\cdot}2°$.

This last finding constituted undeniable proof that the partially racemized acid had been present in these particular tumour hydrolysates, yet we were still puzzled to account for our own results quoted above. We had meanwhile examined the mother liquors from which our first crude crops of hydrochloride had been recrystallized and had been able to obtain evidence for only very small amounts of the *d*-(−) acid. The yield of the natural *l*-(+) acid had however been raised to nearly 10%, and if, as Kögl & Erxleben maintained, we were employing a technique that failed to precipitate the racemic acid as the calcium salt, then the gross amount of glutamic acid in our tissue protein material must have been very much greater than that reported by these workers for the material which they themselves had analysed. It was necessary for us therefore to reinvestigate the problem, using these workers' analytical methods as well as our own.

In the first place we have been unable to confirm Kögl & Erxleben's observation that the calcium salt of the racemic acid has a much greater solubility than that of the natural antipode under the conditions of our modified Foreman analysis. As is shown in the experimental section, both products are equally insoluble. Proof that the racemic acid was indeed precipitated under these conditions was obtained by investigating the rotation of the successive fractions of glutamic acid hydrochloride which we had already obtained from a hydrolysate of wheat gliadin. In this analysis, which has not yet been taken to completion, the first main crop (fraction 1) was salted out with gaseous hydrogen chloride in the usual way and the product recrystallized before the application of our modified Foreman procedure to the collected mother liquors.

The necessary data are given in Table 1. It will be seen that the calcium salts have so far yielded fractions (2–6) representing 9·55% of the protein as the natural *l*-(+) acid and 2·21% as the *d*-(−) acid; in other words 46% of the glutamic acid isolated *via* the Foreman procedure can be regarded as completely

Table 1. *Glutamic acid analysis of wheat gliadin*

Wt. of gliadin 49 g. N = 17·8%	Fraction	Wt. of glutamic acid hydrochloride g.	$[\alpha]_D$ in 9% HCl	Yield % protein: *l*-(+) Glutamic acid	Yield % protein: *d*-(−) Glutamic acid
	1	20·4	+31·4°	33·53	—
	2	4·322	+30·7°	7·13	—
	3	1·862	+ 1·0°	1·59	1·47
	4	0·671	+ 3·4°	0·61	0·49
	5 and 6	0·286	− 2·0°	0·22	0·25
			Total so far isolated	43·08	2·21

racemized. Similar results were obtained with edestin. Here the second and fourth fractions (the third was not available) had rotations of $[\alpha]_D + 23{\cdot}4°$ and $+7{\cdot}3°$ respectively, showing that at least 1·3% of the protein was present in the hydrolysate as the *d*-(−) acid.

These results encouraged us to attempt further analysis of tumour tissue proteins to compare our own procedure with those employed by Kögl & Erxleben. Material prepared from metastatic deposits from the liver of a woman with carcinoma of the colon was analysed by the Foreman method and the results are given in Table 2. It will be seen that the last three fractions show partial racemization (we have adopted Kögl & Erxleben's limit of 30°), but the total amount of *d*-(−) acid present was very small, and more than a third of that suggested is due to fraction 2, a borderline case.

Table 2. *Glutamic acid analysis of protein material from metastases in the liver of a woman with carcinoma of the colon*

Wt. of material 34·5 g. N = 16·2%	Fraction	Wt. of glutamic acid hydrochloride g.	$[\alpha]_D$ in 9% HCl	Yield % protein: *l*-(+) Glutamic acid	Yield % protein: *d*-(−) Glutamic acid
	1	2·92	+31·6°	6·82	—
	2	1·34	+29·9°	3·012	0·088
	3	0·137	+13·2°	0·227	0·093
	4	0·11	+23·0°	0·225	0·035
			Total isolated	10·284	0·216

A companion analysis of 34·5 g. of material by the Dakin butyl alcohol method employed by Kögl & Erxleben when attempting to separate as many of the hydrolysis products as possible gave 3·11 g. of glutamic acid hydrochloride showing $[\alpha]_D + 29{\cdot}0°$ and again the amount of the *d*-(−) acid present was very small, about 0·15% of the protein material. It may be mentioned here that the Foreman analysis ran parallel with those of the three tumour preparations (which could not be taken to completion because the final mother liquors had not been retained) referred to in our earlier communication: in all cases the major part of the glutamic acid was isolated in the first two crops, both of which showed, within reasonable limits, the normal rotation and there can be no doubt that the amounts of *d*-(−) acid in the hydrolysates are small.

We had thus been able to confirm the presence of partially racemized glutamic acid in tumour tissues, but in amounts far short of those claimed by Kögl & Erxleben, so small in fact that we doubted whether approximately the same yields could not be obtained from normal or non-malignant tissues. Using

the Dakin method Kögl & Erxleben had not been uniformly successful in their attempts to isolate glutamic acid hydrochloride from such tissue hydrolysates (see Table 4), and their evidence, the validity of which we discuss below, that it had the normal rotation, was based in part on products isolated by the cuprous oxide procedure of Abderhalden & Fuchs [1908]. Our analyses of normal heart and normal lung tissue proteins are given in Table 3 and it will be seen that the

Table 3. *Glutamic acid analyses of normal ox heart and normal calf lung tissue proteins*

	Fraction	Wt. of glutamic acid hydrochloride g.	$[\alpha]_D$ in 9% HCl	Yield % protein: *l*-(+) Glutamic acid	Yield % protein: *d*-(−) Glutamic acid
Normal ox heart material (47·2 g.) N = 14·3%	1	4·22	+31·4°	7·20	—
	2	1·85	+31·2°	3·16	—
	3	0·522	+30·4°	0·86	—
	4	0·359	+14·9°	0·71	0·16
			Total isolated	11·93	0·16
Normal calf lung material (27·84 g.) N = 15·3%	1	2·70	+31·4°	7·80	—
	2	0·12	+28·8°	0·335	0·015
	3	0·293	+26·5°	0·77	0·063
			Total isolated	8·905	0·078

hydrolysates contained the *d*-(−) acid in amounts comparable with those which we had found in the tumour proteins. It is realized of course that in hydrolysates containing only very small amounts of the *d*-(−) acid the residual solubility of the calcium salt during the Foreman analysis, which cannot be foretold, may operate so as greatly to reduce the amount of the *d*-(−) acid actually isolated, but this will not affect the point at issue, which is the demonstration that the *d*-(−) acid is present in approximately equal amounts in the hydrolysates of both normal and malignant tissue proteins.

As the number of tumour tissues that we had so far examined was small we decided to investigate other samples by the cuprous oxide procedure, which Kögl & Erxleben had themselves used in the majority of cases for the isolation of the glutamic acid hydrochloride. From metastases in liver we readily obtained 5% of glutamic acid showing a rotation of $[\alpha]_D + 30{\cdot}5°$ and from grafted sarcomata of mice (Table 6) 5·0% showing a rotation of $[\alpha]_D + 30{\cdot}1°$, products which again failed to substantiate these workers' claim.

This apparent impasse led us to examine more closely the data, summarized for convenience in Table 4, on which Kögl & Erxleben's claim was based. Analyses 1–8 were carried out by the Dakin method, and, as we have mentioned above, the yields of glutamic acid show very large variations. In analyses 3 and 5, however, both of malignant tissues, large yields were obtained, and, as they emphasize, the products exhibit a high degree of racemization, 89 and 80% respectively (cf. column 7). Using the cuprous oxide method the products isolated from malignant tissues also exhibited racemization in large though varying degree, whereas those from non-malignant tumours and normal tissues did not show this phenomenon. Viewed in this way the evidence for Kögl & Erxleben's claim that racemic glutamic acid is a characteristic of malignancy seems quite convincing. Yet underlying the claim is the assumption that the evidence provided by analyses 8, 9, 10, 11, 14, 23 and 25 is of equal weight to that of analyses 3 and 5. In accepting this, Kögl & Erxleben have relied on the

Table 4. *Glutamic acid analyses of normal and tumour proteins by Kögl & Erxleben*

No.	Material (1)	Wt. g. (2)	Method of analysis (3)	Reference* (4)	Total glutamic acid hydrochloride isolated g. (5)	$[\alpha]_D$ in 9% HCl (6)	Percentage of isolated glutamic acid in *d*-(−) form (7)	Yield % protein: Total glutamic acid (8)	Yield % protein: *d*-(−) Glutamic acid (9)
1	Normal calf lung	15	Dakin	A, Table 1	None isolated	—	—	—	—
2	Rabbit tumour	12·5	,,	A, Table 2	None isolated	—	—	—	—
3	Rabbit tumour	18	,,	A, Table 4	1·85	+ 3·5°	44·5	8·27	3·69
4	Mouse adenocarcinoma	17·7	,,	A, Table 3	None isolated	—	—	—	—
5	Human ovarian carcinoma	48·3	,,	A, Table 5	5·61	+ 3·2°	40·0	9·49	4·16
6	Uterine myoma	85·0	,,	C, Table 1	0·395	+30°	—	0·37	—
7	Uterine myoma	38·2	,,	C, Table 2	None	—	—	—	—
8	Rabbit myxoma	18·7	,,	C	0·092	+20·5°	15·0	0·4	0·06
9	Ovarian tumour	99·5	Cuprous oxide	A, Table 6	1·05	+21·8°	15·6	0·85	0·13
10	Mammary carcinoma	50·9	,,	,,	0·205	+11·6°	31·7	0·32	0·10
11	Mammary carcinoma	20·6	,,	,,	0·156	+16·1°	24·6	0·60	0·15
12	Normal ovary	39·6	,,	,,	0·810	+31·6°	—	1·65	—
13	Normal ovary	2·62	,,	,,	0·104	+31·5°	—	3·20	—
14	Omentum tumour of rabbit	13·66	,,	,,	0·475	+ 4·6°	42·6	2·80	1·20
15	Normal heart from above	3·3	,,	,,	0·16	+31·1°	—	3·90	—
16	Normal lung from above	4·1	,,	,,	0·194	+31·4°	—	3·51	—
17	Normal rabbit heart	3·7	,,	,,	0·136	+31·4°	—	2·96	—
18	Normal rabbit lung	5·1	,,	,,	0·316	+31·3°	—	4·99	—
19	Calf embryo	10	,,	B	0·346	+31·4°	—	2·79	—
20	Calf embryo	15·3	Foreman	B	1·387	+31·5°	—	7·30	—
21	Human myoma	17·6	Cuprous oxide	C	0·595	+28·8°	—	2·72	—
22	Human myoma	17·8	,,	C	0·54	+29·7°	—	2·40	—
23	Human carcinoma, metastases from spleen	13·8	,,	C	0·611	+21·2°	15·1	3·57	0·53
24	Human lung with secondary carcinoma: (1) Normal part	9	,,	C	0·475	+30·2°	—	4·25	—
25	(2) Tumour part	6·5	,,	C	0·395	+24·4°	10·1	4·87	0·54

* A, Kögl & Erxleben [1939, 1]; B, Kögl *et al.* [1939]; C, Kögl & Erxleben [1939, 3].

data given in column 7 and have not considered at all the data we have calculated and give in columns 8 and 9. As protein chemists our attention has throughout been focused on the *yields* of glutamic acid which we could isolate from both malignant and normal tissue protein preparations and when we have attempted to make the separation as quantitative as possible the yields have been uniformly high. Now the cuprous oxide procedure of Abderhalden & Fuchs [1908], which is merely a minor modification of the very early method of salting out the glutamic acid as its hydrochloride from the complete mixture of hydrolysis products by saturation with hydrogen chloride, cannot be quantitative (cf. Tables 5 and 6). It appeared to us therefore that the evidence provided, for instance, by analyses 9, 10 and 11, if interpreted in terms of the data given in column 7, would not provide a valid comparison with the corresponding data from analyses 3 and 5 unless it could be shown that the glutamic acid isolated in such *very small* yields in the former cases was representative of the total amount of this acid present in the hydrolysate, i.e. that it contained at least an average amount of the *d*-(−) antipode. Kögl & Erxleben have assumed that this must be so on the strength of Akkerman's observation that the racemic hydrochloride had a greater solubility in hydrochloric acid than the natural product, which would indicate that the residual acid might be even more completely racemized than the product actually isolated. In our Foreman analyses this relationship undoubtedly held, for the small amount of partially racemized acid was obtained from the later, more soluble, fractions. We remembered however that the material removed as insoluble calcium salt from the hydrolysis mixture by this method consisted essentially of aspartic and glutamic acids and that the major part of the former acid was separated as its copper salt early in the subsequent fractionation; consequently the fact that Akkerman's relationship governed the isolation of glutamic acid hydrochloride from the later mother liquors was to be expected. Yet we were not sure that this would necessarily be so in the cuprous oxide analyses, wherein the glutamic acid hydrochloride was salted out from a very complex mixture of amino-acid hydrochlorides. We had, without difficulty, obtained glutamic acid in yields of 5% by this method and the product had shown no appreciable racemization, yet Kögl & Erxleben, in their analysis 10 (Table 4) had obtained a product showing very complete racemization in a yield as low as 0·32%. Such a yield, moreover, represented only 0·1% of the *d*-(−) antipode, and was thus of the same order as we ourselves had found ultimately in both normal and tumour tissue proteins. This suggested to us the possibility that under the conditions of their cuprous oxide analyses there might have been a preferential salting out of the racemic hydrochloride in the presence of much larger amounts of the natural antipode.

Appropriate experiments showed that this was indeed the case. The conditions of analysis prescribed by Kögl & Erxleben were followed exactly, care being taken to seed the solution with a small crystal of the racemic hydrochloride. In the first experiment, using material from metastases in liver, three successive crops were removed and recrystallized, and as will be seen from the data given in Table 5 it is only the first of these which shows evidence of partial racemization. As this crop represented nearly 1% of the protein material taken for analysis all three crops were returned to the mother liquor and the isolation repeated. We were successful this time in obtaining a first crop, representing only 0·44% of the material, which showed a high degree of racemization. Conditions were then adjusted so as to obtain as large a second crop as possible, in order to permit comparison with our earlier results by this method. The total yield of glutamic acid was 2·83%, very much less than that given by the companion Foreman

Table 5. *Glutamic acid analysis of protein material from metastases in liver of a man with carcinoma of the bronchus*

Method of analysis	Fraction	Wt. of glutamic acid hydrochloride g.	$[\alpha]_D$ in 9% HCl	Percentage of isolated glutamic acid in *d*-(−) form	Yield % protein	
					Total glutamic acid	*d*-(−) Glutamic acid
Cuprous oxide 19 g.: N = 15·5% 1st analysis	1	0·218	20·8°	17	0·93	0·16
	2	0·16	29·0°	4	0·66	0·03
	3	0·27	31·0°	—	1·15	—
Total isolated		0·648	—	—	2·74	0·19
Same material: 2nd analysis	1	0·103	11·6°	31·7	0·44	0·14
	2	0·565	31·0°	—	2·39	—
Total isolated		0·668	—	—	2·83	0·14
Foreman 39 g.	1	3·88	31·4°	—	8·04	—
	2	0·572	26·5°	8	1·18	0·074
	3	0·326	29·5°	3·5	0·67	0·023
	4	0·178	19·8°	19	0·36	0·068
Total isolated		4·956	—	—	10·25	0·165

analysis. Analogous results were given by protein material from grafted sarcomata of mice (Table 6), although in this case the degree of racemization observed in the first crop of glutamic acid hydrochloride was not so pronounced. Likewise with normal heart muscle the rotation of the first small crop of hydrochloride was fairly high $[\alpha]_D + 29°$. These variations may be merely reflecting the small amount of the *d*-(−) antipode in the hydrolysate concerned; on the other hand it must be remembered that the solubility of the racemic hydrochloride, like that of any other amino-acid or its salt, must be a function of the dielectric constant of the medium [cf. Cohn, 1935], and in the case of protein hydrolysates this will depend on the particular amino-acid mixture present therein, a point that may be lost sight of by workers inexperienced in protein analysis.

Table 6. *Glutamic acid analysis of protein material from grafted sarcomata of mice*

Method of analysis	Fraction	Wt. of glutamic acid hydrochloride g.	$[\alpha]_D$ in 9% HCl	Percentage of isolated glutamic acid in *d*-(−) form	Yield % protein	
					Total glutamic acid	*d*-(−) Glutamic acid
30 g.: N = 15·0% 1st analysis by cuprous oxide	1	1·85	+30·3°	—	4·97	—
Repeat analysis of same material	1	0·1468	+28·3°	5·4	0·39	0·02
	2	0·3988	+31·0°	—	1·07	—
Foreman analysis on mother liquor of above	3	2·24	+31·4°	—	6·01	—
	4	0·323	+24·8°	9	0·87	0·094
Total isolated		3·1086	—	—	8·34	0·114

The results given in Table 5 are most instructive. Fraction 1 of the second cuprous oxide analysis is very similar to the analysis 10 (Table 5) of Kögl & Erxleben mentioned above; both samples of glutamic acid hydrochloride show the same high percentage racemization (63·4%), yet the other data given in Table 5 leave no doubt that fraction 1 is in no way representative of the re-

maining glutamic acid of the hydrolysate. Indeed the maximum amount of the *d*-(−) antipode present in the whole hydrolysate was no more than about 0·2% of the protein material, so that the percentage racemization of the total glutamic acid was no more than 4%. Such, admittedly somewhat unexpected, findings provide a complete explanation of the many anomalous results obtained by other workers besides ourselves [cf. White & White, 1939; Arnow & Opsahl, 1939; Graff, 1939]. In those cases in which the dicarboxylic acids have been separated by Foreman's method the first two or three crops of glutamic acid hydrochloride will show very little, if any, racemization if the natural antipode be present in large excess, whereas the product salted out from the complete hydrolysis mixture of amino-acids (cuprous oxide) or an attenuated mixture (Dakin), provided the yield be small enough, may exhibit considerable racemization. Similar reasoning also explains the results of Kögl, Erxleben & Akkerman's experiments in which the racemic acid had been added to the hydrolysate of normal calf embryo, for the single-step Foreman analysis which they performed has undoubtedly failed to reveal the maximum amount of the natural antipode originally present therein.

Reverting finally to Kögl & Erxleben's claim that partially racemized glutamic acid is a characteristic of malignancy we see at once that the interpretation by them of the data given in Table 4 in terms of percentage racemization of the isolated product (column 7) is not a valid procedure. If comparison of their cuprous oxide analyses with our own can be taken as a guide, then, in the absence of definite evidence to the contrary, we must assume that their yields of the *d*-(−) antipode (column 9), which show wide variations as between one tumour and another, will not be far short of the total amounts present in the respective hydrolysates. The yields in two cases (analyses 3 and 5) are indeed extremely large, while in another (analysis 14) the yield is larger than we have encountered, but in the remainder it is of the same order of magnitude as we ourselves have found, not only in hydrolysates of seven different samples of tumour protein material but also, if appropriate analytical methods be employed, in those of two normal animal proteins as well. The sharp differentiation which Kögl & Erxleben thought they had established as between normal and non-malignant tissue proteins on the one hand and malignant tissue proteins on the other hand is thus no longer valid and since the grafted sarcomata of mice which we examined (Table 6) consisted almost wholly of malignant tissue there can be no doubt that the occurrence of *d*-(−)glutamic acid is not in any way a characteristic of malignancy.

The early masters of protein analysis, Emil Fischer and T. B. Osborne, always advised against the comparison of proteins on a basis of amino-acid composition, for protein analyses were then, as they remain in most cases to-day, far from being quantitatively complete. This criticism can be applied to Kögl & Erxleben's more extensive analyses of certain of their samples of normal and tumour protein material, for although most of the known protein amino-acids have been isolated, the overall yields represent, even in the most favourable cases, no more than half the protein material taken for analysis. Moreover, they were not comparing one particular protein with another; for both normal and malignant tissues contain a mixture of these substances, the individual members of which will be different in each particular normal or malignant tissue concerned. Consequently their various tissue hydrolysates may have contained very different amino-acid mixtures, a position which might profoundly affect the yield of an amino-acid separated by a method involving the solubility of derivatives such as copper salts. Copper aspartate, for instance,

is insoluble in cold and nearly insoluble in boiling water, yet during our analysis of various pure proteins for dicarboxylic acids we have frequently obtained fractions of copper salts freely soluble in cold water from which we have subsequently isolated, after appropriate treatment, 30–40 % of copper aspartate. Such experience leads us to doubt whether any importance can be attached to the isolation by Kögl & Erxleben from samples of malignant tissue protein material of, for instance, leucine and valine exhibiting a few per cent racemization until evidence is produced to show that the yields in these and *in the comparative analyses of normal tissue material* are well nigh quantitative, for the conditions governing the solubilities of the lead and copper salts of the racemized acids during the fractionation of the hydrolysis products may have been different in the two cases, thus masking their presence in the normal tissue.

Table 7. *Aspartic acid analyses of normal and malignant tissue protein material*

Material	Fraction	Wt. of copper salt g.	$[\alpha]_D$ in 10 % HCl	Yield % protein: Total aspartic acid	Yield % protein: *d*-(+) Aspartic acid
Normal heart muscle (47·2 g.) (compare Table 3)	1	4·533	+25·6°	4·64	—
	2	0·59	+24·0°	0·90	0·03
	3	0·29			
	4	0·566	+ 0·7°	0·58	0·29
	Total isolated	5·979	—	6·12	0·32
Metastases from liver (34·5 g.) (compare Table 2)	1	3·98	+25·7°	5·57	—
	2	0·64	+24·6°	0·91	—
	3	0·346	+ 4·5°	0·50	0·20
	Total isolated	4·966	—	6·98	0·20

An interesting illustration of this is afforded by a comparison of Kögl & Erxleben's and our own analyses for aspartic acid. The former workers' yields from both malignant and normal tissue materials were low and the products isolated showed no racemization; our own yields, on the contrary, we believe to be wellnigh quantitative (Table 7) and although the first and largest fraction in each case showed no racemization there was clear evidence of this in the later ones. We have thus another instance of non-correlation between racemization and malignancy.

Kögl & Erxleben admit that the "unnatural" *d*-amino-acids might be constituents of the protein molecule of a normal tissue and emphasize that their occurrence must not be regarded *a priori* as "pathological". But in these cases they suggest that the *d*-amino-acids are to be regarded as being built into the molecule in a "sensible" (*sinnvolle*) way as opposed to the "senseless" (*sinnlose*) way in which they become incorporated in the protein molecule of cancer tissues. By this they mean, we presume, that the 2·2 % of *d*-(−)glutamic acid we find in wheat gliadin and the 0·16 % we find in normal heart tissue protein are to be regarded as "normal constituents" of the proteins concerned, whereas the 4·0 % they have found in two malignant tissue proteins are evidence of some "disorder" in the protein metabolism. The data given in the present paper show that such speculations are as yet uncalled for, since the high yields in the latter cases appear to be themselves abnormal for the tissues concerned. Protein chemists have so far given but little attention to the occurrence of racemized amino-acids in protein hydrolysates and it may well be that they are more widespread

than has been imagined, for the possibility that racemization accompanies the splitting of certain peptide bonds during hydrolysis of the protein molecule must not be lost sight of.

Experimental

As we pointed out in our earlier communication [Chibnall *et al.* 1939], the hydrolysis procedure of Kögl & Erxleben (7 hr. with conc. HCl) does not effect the maximum splitting of peptide bonds and in our modified Foreman analyses quoted below we have preferred the more usual hydrolysis with 20% HCl for 20–24 hr. Appropriate experiments have shown that when pure *l*-(+)glutamic acid is submitted to such hydrolysis and then passed through the complete Foreman procedure no appreciable racemization occurs. The crystalline hydrochloride recovered represented 96·1% of the original glutamic acid.

In the analyses described below the purity of the various crops of recrystallized glutamic acid hydrochloride was controlled by determining total N, and by confirming that the titration value in the micro-Kjeldahl was exactly one-half of that obtained when the same weight of material in aqueous solution was titrated to *p*H 7·3 (blue tint with bromothymol blue). To avoid repetition in the text it may be stated that all rotations of glutamic acid hydrochloride were observed in 9% HCl and calculated as glutamic acid.

Solubility of racemic calcium glutamate under the conditions of a modified Foreman analysis. Kögl, Erxleben & Akkerman's [1939] procedure was followed exactly, although the aqueous volume from which the Ca salts were precipitated was about one-tenth of that normally used in our protein analysis. A mixture of 756·8 mg. of *l*-(+)glutamic acid and 824·3 mg. of synthetic racemic glutamic acid ($+H_2O$), giving, by calculation, a rotation of $[\alpha]_D + 15·6°$, were dissolved in 55 ml. of warm water and freshly prepared calcium hydroxide cream was added in excess. The solution was filtered, the residue washed with warm water and the collected filtrate reduced *in vacuo* to 6·5 ml. when a small amount of Ca salt separated. Without removing this material 60 ml. of 95% alcohol were added, the precipitated Ca salts filtered off on a sintered glass crucible and washed with a few ml. of absolute alcohol. The Ca salts were redissolved with difficulty, about 150 ml. of boiling water being required. The small insoluble residue, presumably mostly $CaCO_3$ derived from the excess $Ca(OH)_2$ precipitated from the original aqueous solution by the alcohol, was filtered off; analysis showed that this contained only 0·138 mg. N. The aqueous solution was concentrated as before and the precipitation repeated. Freed as it was from a small excess of lime the precipitate was this time very insoluble, and 600 ml. of boiling water were required to redissolve it. A small residue was filtered off (N = 0·4 mg.) and Ca removed from the filtrate while this was still warm. The precipitated Ca oxalate was washed four times by boiling with portions of 150 ml. of water and centrifuging, the collected liquors reduced *in vacuo* at 40° to about 150 ml., the final adjustment for the removal of Ca made with oxalic acid, and the filtered solution concentrated *in vacuo* to 70 ml. A few ml. of HCl were then added and the solution taken finally to dryness in a vacuum desiccator. The glutamic acid hydrochloride recovered weighed 1·872 g. and it showed a rotation of $[\alpha]_D + 15·6°$. When the material was dissolved in water there remained a small residue, about 2%, of Ca oxalate; this was filtered off, the volume adjusted (warm) to 10 ml. of 20% HCl and the solution allowed to crystallize at room temperature. 1·37 g. of hydrochloride were recovered; $[\alpha]_D + 19·8°$. The two aqueous-alcoholic filtrates were taken to dryness and submitted to Kjeldahl analysis: 1·57 mg. of N were found, corresponding to just over 1% of that present in the original mixture of

glutamic acids. The large loss encountered by Kögl & Erxleben cannot therefore be attributed to the solubility of either the racemized or the *d*-(−) antipode Ca salt and it must have been due to incomplete re-solution of the Ca salts or to crystallization of these from solution during removal of Ca if this operation had been performed in the cold. It may be remarked that in a protein analysis by our normal procedure we have encountered no difficulty in redissolving the Ca precipitates, owing no doubt to the presence of the $CaCl_2$ arising from the combined HCl of the amino-acid hydrochlorides in the hydrolysates.

Protein material from normal ox heart

Fresh ox heart was washed carefully to remove blood and finely minced. 1200 g. of the minced material were extracted twice with 3 l. of ice-cold 5% trichloroacetic acid and then washed three times with 2 l. of acetone. The residue was dried at 50° and then extracted thoroughly, first with boiling 95% alcohol and finally with ether.

Foreman analysis. To illustrate the intricacies of the modified Foreman procedure we have used in the present research we shall describe this ox heart protein analysis in full. Brief details only will be given of the remaining analyses, wherein minor modifications were occasionally introduced as circumstances warranted.

47·2 g. of dry material were hydrolysed with 6 vol. of 20% HCl for 22 hr. The excess HCl was then removed in the usual way by repeated evaporation to a syrup *in vacuo*, cream of lime added and the excess lime, together with the humin, removed by centrifuging. The lime-humin residue was washed four times with 150 ml. of hot water and the collected liquors evaporated *in vacuo* to remove ammonia. The volume was then made up to 400 ml. and 3420 ml. of absolute alcohol added with vigorous stirring. The separated Ca salts were filtered off without delay on a Büchner funnel and the extraneous mother liquor removed by pressing the mass to a hard pad, which was then washed with a small volume of absolute alcohol. On redissolving the material in about 300 ml. of warm water a small residue, presumably $CaCO_3$, remained undissolved. Without removing this, 10 ml. of cream of lime were added, the volume adjusted to 400 ml. and the precipitation with alcohol repeated. The washed Ca salts were dissolved out with 800 ml. of warm water, the small residue washed four times by stirring with 150 ml. of water kept near the boil for a few minutes, centrifuged and the clear liquors collected. Ca was removed with oxalic acid without delay to prevent crystallization of the relatively insoluble Ca salts; the Ca oxalate was washed four times with 150 ml. of hot water as described above, the mixture cooled to room temperature and kept for some time to permit recrystallization of the small amounts of oxalate redissolved. The oxalate was then removed by centrifuging, the clear liquors collected and reduced *in vacuo* to about 80 ml. Final adjustment to remove residual Ca or oxalic acid was then made, the precipitate centrifuged off and washed as before, and about 20 ml. of HCl added to the clear liquors which were then evaporated to a syrup. On standing overnight at room temperature much glutamic acid hydrochloride crystallized out. This was removed, redissolved in about 20 ml. of water, 5 ml. of HCl were added and the mixture concentrated in a dish on the water bath to incipient crystallization. Next day the material was filtered off on a sintered glass filter, washed with HCl and dried over NaOH in a vacuum desiccator. This first crop of glutamic acid hydrochloride weighed 4·22 g. and the rotation was $[\alpha]_D + 31{\cdot}4°$ (fraction 1, Table 3). By treatment of the mother liquors a

LINDA HALL LIBRARY
KANSAS CITY, MO.

second crop of hydrochloride was obtained, which was recrystallized as before. The weight was 1·852 g. and the rotation $[\alpha]_D + 31·2°$ (fraction 2, Table 3).

The mother liquors were again collected, excess HCl removed by repeated evaporation *in vacuo* to a syrup, which was then taken up in 500 ml. of water, boiled with sulphate-free $CuCO_3$, the excess removed by filtration on a hot Büchner funnel and the filtrate and washing reduced *in vacuo* to 170 ml. On standing at room temperature overnight a large amount of greenish-blue, and therefore impure, Cu aspartate separated. This was filtered off, suspended in 500 ml. of water kept near the boil, HCl added drop by drop until solution was complete, $CuCO_3$ added and the excess removed by filtration on a hot Büchner funnel. The clear filtrate was kept overnight, when 4·533 g. of pure (blue) crystalline Cu aspartate separated.[1]

Cu was removed from the first Cu filtrate with H_2S, excess HCl removed by evaporation in the usual way and a third Foreman precipitation of the Ca salts made from an aqueous volume of 100 ml. On treatment as before there was obtained a third crop of glutamic acid hydrochloride weighing 0·7498 g. Recrystallized from 20 % HCl this gave a crop of 0·654 g., the mother liquor being reserved for treatment as described below. A small amount of difficultly soluble inorganic material was removed from this crop by repeated solution in a small volume of water and filtration, when there was finally obtained 0·5222 g. of hydrochloride with a rotation of $[\alpha]_D + 30·4°$ (fraction 3, Table 3).

The Cu treatment was next applied to the main mother liquor when two crops (0·59, 0·29 g.) of pure recrystallized Cu aspartate were obtained. Following this, Cu was removed in the usual way and a fourth crop of glutamic acid hydrochloride (0·1762 g.) obtained. The filtrate was then again passed through the Foreman procedure (aqueous volume 25 ml.) to give a fifth crop (0·2836 g.) of hydrochloride. The filtrate from this was given another Foreman precipitation (aqueous volume 20 ml.) and the solution of free amino-acids recovered from the Ca salts evaporated to a small volume *in vacuo*. On standing 0·1232 g. of crude tyrosine separated. The mother liquor was then concentrated slowly to a syrup in a vacuum desiccator and after 2 days a few drops of alcohol were stirred in. On standing, 0·78 g. of material separated; from 0·4 g. of this a fourth crop of Cu aspartate (0·27 g.), but no glutamic acid, was separated. The ultimate mother liquor, on treatment with HCl, finally gave a sixth crop (23 mg.) of glutamic acid hydrochloride. Crops 4, 5 and 6, with the mother liquor from the recrystallization of crop 3, were collected, dissolved in about 2–3 ml. of cold water, filtered to remove an insoluble inorganic residue and the filtrate reduced to dryness *in vacuo*. This treatment was repeated until a clear solution was obtained without filtration. On crystallization from 20 % HCl there was then obtained 0·358 g. of glutamic acid hydrochloride (fraction 4, Table 3) with a rotation of $[\alpha]_D + 14·9°$. It may be mentioned here that contamination of the later fractions of the hydrochloride with inorganic material was always observed in the case of the crude tissue hydrolysates discussed in the present paper, but we have not so far encountered it with corresponding fractions from pure proteins. As will be seen from Table 3, the gross yield of glutamic acid was 11·93 % and of the *d*-(−) antipode 0·16 %; 2·6 % of the glutamic acid can therefore be regarded as fully racemized.

Aspartic acid was recovered from the Cu salt by dissolving in the necessary volume of boiling water and removing Cu with H_2S. The recovery of crystalline

[1] The purification of these crude Cu salts needs experience, as the impurities present may separate either before or after the Cu aspartate during recrystallization. We shall refer to this more fully in a later paper.

acid was between 95 and 100%. The product obtained from the first crop of Cu salt had the normal rotation of the *l*-(−) antipode in 10% HCl of $[\alpha]_D + 25{\cdot}6°$; that from the second and third crops combined had $[\alpha]_D + 24°$, while that from the fourth crop showed $[\alpha]_D + 0{\cdot}7°$. The overall yield of aspartic acid was 6·12% and of *d*-(+) antipode 0·32%; 10·5% of the total acid can therefore be regarded as fully racemized (Table 7).

Abderhalden & Fuchs (cuprous oxide) analysis. Kögl & Erxleben's procedure, certain points of which are emphasized in the first malignant tumour analysis described below, was followed. From 28·3 g. of protein material a first recrystallized crop (0·12 g.) of glutamic acid hydrochloride was obtained which showed $[\alpha]_D + 29°$. Repetition of the procedure several times did not give a first crop showing any higher degree of racemization.

Protein material from normal calf lung

Fresh calf lung (500 g.) was finely minced and extracted with 1500 ml. of ice-cold 5% trichloroacetic acid. The material was again treated in the same way and then washed three times with 1 l. of acetone. The residue was dried at 50° and then thoroughly extracted, first with boiling 95% alcohol and then with ether.

27·8 g. of dry material were hydrolysed with 6 vol. of 20% HCl for 22 hr., and then passed through the Foreman procedure detailed above. Data are given in Table 3. After the initial double precipitation (aqueous volume 200 ml.) 3·255 g. of crude hydrochloride were separated; on recrystallization this gave two crops, 2·70 and 0·12 g., showing $[\alpha]_D + 31{\cdot}4°$ and $+28{\cdot}5°$ respectively (fractions 1 and 2, Table 3). A first crop of crude Cu aspartate was then removed; on recrystallization this gave 2·26 g. of pure Cu aspartate. A third Foreman precipitation (aqueous volume 25 ml.) gave a third crop (0·288 g.) of glutamic acid hydrochloride, but no insoluble Cu salt could be obtained. A fourth Foreman process (aqueous volume 15 ml.) gave a fourth crop (70 mg.) of hydrochloride and a small second crop (0·4 g.) of recrystallized Cu aspartate. The mother liquor, on standing at room temperature for 14 days, slowly deposited a third crop (0·17 g.) of Cu aspartate. The third and fourth crops of hydrochloride were combined with a small crop (37 mg.) obtained from the mother liquor of crop one; inorganic material was removed as before and 0·293 g. of material showing $[\alpha]_D + 26{\cdot}5°$ obtained (fraction 3, Table 3). The overall yield of glutamic acid was 8·9%, of the *d*-(−) antipode 0·078% and of aspartic acid (not further characterized) 4·92%: 1·8% of the total glutamic acid isolated can thus be regarded as completely racemized.

Protein material from metastases in liver of a man with carcinoma of the bronchus

Malignant nodules from the liver of a man (W. V. aged 55 years) who had died from carcinoma of the bronchus were separated from the liver tissue. The material (2500 g.) was minced and extracted in the cold with 10 l. of 0·6% NaCl. 4 vol. of 90% alcohol were added to the separated extract, and the precipitated protein dried at 50° (108 g.). The residue after extraction was washed with 80% alcohol and dried at 100° (260 g.). The two products were combined, finely ground, washed with warm water until free from NaCl, then repeatedly with boiling 95% alcohol and finally with ether.

Abderhalden & Fuchs (cuprous oxide) analysis. 19 g. of dry material were hydrolysed for 7 hr. with three times the weight of HCl (sp. gr. 1·19) and then treated exactly as described by Kögl & Erxleben [1939, 1]. The pale yellow

hydrolysate thus obtained was concentrated to a thick syrup (20 ml.) in a 50 ml. centrifuge tube, seeded with a small crystal of the racemic hydrochloride and kept in the ice box at 0°. During the course of 2 days a mass of large crystals appeared around the nucleus in the upper layer of the syrup, together with a much finer white crystalline flocculum which was dispersed throughout the remainder of the syrup. The separated solid was then filtered off on a sintered glass filter, washed with a few drops of HCl and dried *in vacuo* over NaOH. The material (0·2927 g.; $[\alpha]_D$ +20·4°) was recrystallized from 1 ml. of 20% HCl (0·2183 g.; $[\alpha]_D$ +20·8°). The collected mother liquors were treated twice successively in the same way, and further crops of recrystallized material obtained. (Data given in Table 5, 1st analysis.)

To obtain, if possible, a first crop showing more complete racemization, all the materials of this first analysis were collected, returned to the mother liquor and the treatment repeated. As before, large crystals formed around the nucleus in the upper layer of syrup and a white flocculum appeared throughout the rest of the liquid. On centrifuging, these crystals remained at the top, while the flocculum collected at the bottom of the clarified liquid, and it was possible to filter off the former without contamination with the latter. The crop weighed 0·1274 g. and showed $[\alpha]_D$ +9·5°. Recrystallized from 1 ml. of 20% HCl this gave 0·103 g. showing $[\alpha]_D$ +11·6°. The mother liquors were collected, treated as before, and left for 2 days at 0°. The recrystallized material weighed 0·564 g. and showed $[\alpha]_D$ +31·0° (see Table 5, 2nd analysis).

Foreman analysis. The conditions of this analysis followed closely those described above for normal lung tissue material, and the data for the four fractions of glutamic acid hydrochloride obtained are given in Table 5; 2·8% of the total glutamic acid isolated can be regarded as completely racemized. Two crops of recrystallized Cu aspartate were obtained (5·22 g. and 0·18 g.), and the yield of aspartic acid (not further characterized) was 6·69%.

Protein material from metastases in liver of a woman with carcinoma of the colon

Metastatic deposits (750 g.) from the liver of a woman (E. B. aged 73 years) with carcinoma of the colon were minced and extracted with 3 vol. of 3% trichloroacetic acid. The insoluble material was washed once with the same reagent, then three times with acetone and dried. The material was finely ground, exhaustively extracted with boiling 95% alcohol and then with ether.

Dakin (butyl alcohol) analysis. 34·5 g. of dry material were hydrolysed for 7 hr. with three times the weight of HCl (sp. gr. 1·17) and passed through the procedure outlined by Kögl & Erxleben [1939, 1]. Three successive crops of crude hydrochloride were separated and the collected material recrystallized from 20% HCl. 3·11 g. of glutamic acid hydrochloride were thus obtained, showing $[\alpha]_D$ +29·0°. The yield of glutamic acid (7·26% and at the most only 0·2% of the *d*-(−) antipode) was lower than that of the companion Foreman analysis given below, owing undoubtedly to incomplete hydrolysis, and perhaps in part to the conditions under which the monoamino-acids were extracted not being optimal, leading to loss of dicarboxylic acids from the aqueous solution [cf. Sharp, 1939].

Foreman analysis. 34·5 g. of dry material were hydrolysed for 20 hr. with 6 vol. of 20% HCl. The analysis followed closely those described above for normal heart material, and the data for the glutamic acid isolated are given in Table 3. It will be seen that 4·3% of the isolated glutamic acid can be regarded

as completely racemized. After the second and third Foreman precipitations crops of recrystallized Cu aspartate were removed. Following the fourth precipitation a crystalline crop (0·4738 g.) of amino-acids was obtained by evaporation of the Ca-free filtrate; from this 0·346 g. of Cu asparate was separated. Data for the aspartic acid recovered from the three crops of salt are collected in Table 7. It will be seen that the yield of aspartic acid was 6·98 % and of the *d*-(+) antipode 0·20 %, so that 5·6 % of the acid can be regarded as completely racemized.

Protein material from grafted sarcomata of mice

Grafted sarcomata of the strain M.C.D.B. 1 (see Boyland & Boyland [1939]) were removed from mice and finely minced. The material (240 g.) was extracted twice with 3 % trichloroacetic acid and the residue washed twice with acetone and dried. The product was finely ground and exhaustively extracted first with boiling 95 % alcohol and then with ether.

30 g. of dry material were hydrolysed with three times the weight of HCl (sp. gr. 1·17) for 7 hr. and the cuprous oxide procedure applied. In the first separation 1·85 g. of recrystallized glutamic acid hydrochloride were obtained, showing $[\alpha]_D + 30·3°$ (Table 6, 1st analysis). The material was returned to the mother liquor and conditions adjusted (as described above) to yield two small successive crops. On recrystallization these weighed 0·1482 and 0·3988 g., showing $[\alpha]_D + 28·3°$ and $+31·0°$ respectively (Table 6, 2nd analysis). The collected mother liquors were then given a double Foreman precipitation (aqueous volume 250 ml.) and two crops (2·05, 0·296 g.) of crude hydrochloride removed. These were combined and recrystallized from 20 % HCl to give 2·24 g. of glutamic acid hydrochloride: $[\alpha]_D + 31·4°$ (Table 6, fraction 3); 2·29 g. of Cu aspartate were next obtained, followed by a third crop (0·2 g.) of crude hydrochloride. Since much intractable material remained in the mother liquors at this stage they were hydrolysed with 20 % HCl for 20 hr., followed by a single Foreman precipitation (aqueous volume 20 ml.). A fourth crop of crude hydrochloride (0·192 g.) was then removed, next 0·763 g. of Cu aspartate and finally a fifth crop (38 mg.) of crude hydrochloride. Crops 3, 4 and 5 were collected, insoluble inorganic material removed as described before, and the material recrystallized from 20 % HCl; 0·323 g. of glutamic acid hydrochloride was thus obtained, showing $[\alpha]_D + 24·8°$ (Table 6, fraction 4). The overall yield of glutamic acid was 8·34 %, of the *d*-(−) antipode 0·114 %, and of aspartic acid 4·96 %: all of them will be less than the maximum obtainable as complete hydrolysis was not attained until much material had been rejected by the Foreman treatment.

SUMMARY

The claim of Kögl & Erxleben that racemization of amino-acids, especially of glutamic acid, is a characteristic of the proteins of malignant tissues has been investigated in some detail.

Quantitative isolation of glutamic acid by a modification of Foreman's method from seven different malignant tissue protein preparations showed that in all cases the yield was high (*ca.* 10 %) and that the degree of racemization was small, the yield of the *d*-(−) antipode being only about 0·2 %. Similar results were obtained from two normal tissue protein preparations; gliadin, a plant protein, gave 2·2 % of the *d*-(−) antipode.

The cuprous oxide (Abderhalden & Fuchs) method used by Kögl & Erxleben to isolate glutamic acid as its hydrochloride from tissue hydrolysate has been shown to give the racemized product preferentially, even when a large excess

of the *l*-(+) antipode is present. The interpretation by Kögl & Erxleben of their results obtained by this method is thus shown to be invalid.

It is shown that small amounts of partially racemized aspartic acid, as well as of glutamic acid, can be isolated from both normal and malignant tissue protein material and it is concluded that racemization of amino-acids is not a characteristic of the proteins of malignancy.

We are indebted to Dr W. G. Barnard, Pathologist in charge of the Central Histological Laboratory of the London County Council, and to Dr L. M. Hawksley, Pathologist of the Royal Cancer Hospital, for supplying and identifying the human malignant tissues used. We should also like to thank Dr H. A. Krebs for a sample of *d*-(−)glutamic acid and Dr Paul Fildes for a sample of racemic glutamic acid. We would like to thank the British Empire Cancer Campaign for their support in this research.

REFERENCES

Abderhalden & Fuchs (1908). *Hoppe-Seyl. Z.* **57**, 339.
Arnow & Opsahl (1939). *Science*, **90**, 257.
Boyland & Boyland (1939). *Biochem. J.* **33**, 618.
Chibnall, Rees, Tristram, Williams & Boyland (1939). *Nature, Lond.*, **144**, 71.
Cohn (1935). *Ann. Rev. Biochem.* **4**, 93.
Foreman (1914). *Biochem. J.* **8**, 463.
Graff (1939). *J. biol. Chem.* **130**, 13.
Kögl & Erxleben (1939, 1). *Hoppe-Seyl. Z.* **258**, 57.
—— —— (1939, 2). *Nature, Lond.*, **144**, 111.
—— —— (1939, 3). *Hoppe-Seyl. Z.* **261**, 154.
—— —— & Akkerman (1939). *Hoppe-Seyl. Z.* **261**, 141.
Sharp (1939). *Biochem. J.* **33**, 679.
White & White (1939). *J. biol. Chem.* **130**, 435.

43. THE PROTEINS OF *HEVEA BRASILIENSIS*

1. ANALYSIS OF A PRODUCT ISOLATED FROM DRIED LATEX

By G. R. TRISTRAM

From the Department of Biochemistry, Imperial College of Science and Technology, London, S.W. 7

(*Received 30 January 1940*)

Much work has been done on the correlation between the physical properties and the protein content of rubber latex. Weber [1903] and Freundlich & Hauser [1925] suggested that the rubber globules in latex were coated with protein and that this protective layer influenced the coagulation of the latex [see also Bondy & Freundlich, 1938]. Knowledge regarding the nature and composition of the protein(s) of latex, however, is still incomplete, and with the exception of the work of Belgrave [1925] and Bishop [1927] and the early work of Frank [1914] there have been no detailed accounts of the isolation and differentiation of the individual proteins.

Some attention must be given to the work of Belgrave and Bishop because, apart from qualitative tests mentioned in other papers, they are the only workers who have attempted to elucidate the chemical composition of the proteins of latex. Thus Belgrave [1925] obtained a single product by a method involving the use of alcohol as a rubber precipitant, while Bishop [1927] isolated three products, A, B and C, from serum obtained by the freezing of fresh latex. The results, obtained by the indirect method of Van Slyke, are given in Table 1.

Table 1

Values quoted are in percentage of total protein-N

	Belgrave [1925]	Bishop [1927]		
	1	A (acid)	B (NaCl)	C (heat)
Ammonia-N	7·7	5·65	1·62	5·0
Humin-N	0·5	0·45	0·29	0·14
Diamino-N	34·6	22·6	54·0	10·0
Non-basic-N	59·2	56·9	41·8	83·4
Total	102·0	85·6	97·7	98·5

It can be seen that none of Bishop's results agrees with those of Belgrave, nor would it appear that any combination of them would agree.

The results obtained by Belgrave are of particular interest. From 11 l. latex (D.R.C. 38–40%) he isolated 8·5 g. protein (N, 11·6%). This is equivalent to 1·5 g. protein (N, 15%) per kg. of dry weight (total solids *ca.* 40%), which amount corresponds very closely to that isolated, under the best conditions, by the present author. Moreover, recent work [Miller, 1935; Tristram, 1939] has shown that the Van Slyke method gives values for basic N which may be misleading, and on these grounds the diamino-N of 34·6% reported by Belgrave might well represent a true basic N of approximately 20%, a value found by direct analysis in the present work. Table 2 shows the comparison between the two products.

Table 2

	Belgrave	Author
Yield per kg. D.R.C.	1·5 g.	1·0–1·5 g.
Total N %	11·6	14·95
Humin-N (% of total N)	0·5	3·03
Basic-N (% of total N)	34·6	20·0
Ammonia-N (% of total N)	7·7	7·1

Apart from a recent paper by Midgeley *et al.* [1937] little is known of the amino-acid composition of the proteins of latex, and these workers do not mention the amounts of the various amino-acids found in the protein analysed. The product isolated by them from rubber by fractionation with organic solvents contained 12·5% N, and they obtained evidence of the presence of glycine, aspartic acid, leucine, proline, arginine, histidine and lysine; while cystine, tyrosine and glutamic acid were reported as being definitely absent. Such an analysis cannot be regarded as satisfactory, and it was decided that a more detailed study of the amino-acid composition might furnish valuable data on the products likely to result from degradation of the protein during various technical processes. At the same time it was hoped that comparative analyses might indicate how many proteins exist in latex.

Experimental

The chief difficulty with regard to the preparation of one, or perhaps more than one, protein from latex lay in the fact that the fresh latex itself was not available, and after experiments with various preserved products it was ultimately decided to use air-dried films prepared in Malaya from fresh latex as the starting material. It was realized that drying may have caused some denaturation but whatever the change it should not have affected the amino-acid composition of the protein(s).

The method employed was to extract the film with a borate buffer, *p*H 9·2. The dried latex was shredded into thin strips and extracted for 12 hr. (including 3 hr. at 50°) with 0·024 *M* sodium diborate [cf. Lugg, 1939] to which were added 0·4 vol. of alcohol and 0·1 vol. of ether. The extract was filtered through paper pulp with a top layer of silica gel (B.D.H. Hyflo Super Cel) and the protein coagulated by acidifying to *p*H 4·5–5·0 with 10% acetic acid. The yield was found to be very variable.

In one experiment approximately 24% of the total N of the dried latex was extracted, and of this amount about 30% was precipitated as protein-N, corresponding to 1·2–1·9 g. protein per kg. of dried latex. An attempt was made to obtain increased yields of protein by repetition of the borate treatment after re-shredding the extracted strips on smooth rolls. Much nitrogenous material was thus obtained in solution but the amount of protein therein was negligible. Whether any protein was rendered unavailable by this latter treatment is uncertain, but it is known that mastication does, in fact, produce a change in the protein and it may be that all, or part, of that which remained in the films was denatured on the rolls and could not, in consequence, be extracted by the borate buffer, which, according to Dr J. W. H. Lugg (private communication), will not extract denatured protein. Various other media were tried, including solutions of LiCl, NaCl, Na_2SO_4 and $(NH_4)_2SO_4$, but none of these extracted any protein.

The protein obtained was perfectly white while wet, but on removing water by means of alcohol it darkened somewhat to a buff powder. The isoelectric

point was not determined accurately, but flocculation from borate solution occurred between *p*H 4·5 and 5·0. The undehydrated product was soluble in dilute NaOH (*p*H 8·5), and was precipitated from such a solution by nearly complete saturation with NaCl (the precipitate did not redissolve on dilution), and by one-third saturation with $(NH_4)_2SO_4$. (Bondy & Freundlich [1938] isolated from ammoniated latex a protein which was precipitated from solution by one-third saturation with $(NH_4)_2SO_4$.)

Surface films

A sample of protein which had not been extracted with alcohol and ether was dissolved in *N*/20 NaOH and diluted with borax to make an approximately 0·01 % solution of *p*H 8·5. This solution was spread on phosphate buffer (*p*H 7·0) and examined by means of a Langmuir-Adam surface pressure balance. On compression the pressure became measurable at a surface concentration of *ca.* $0{\cdot}4 \times 10^{-7}$ g. protein per cm.^{2}, and rose steadily to reach a value of 14 dynes/cm. at a concentration of *ca.* $0{\cdot}8 \times 10^{-7}$ g./cm.^{2} The film was liquid throughout. The areas observed are much smaller than those normally found for proteins [Adam, 1938, p. 87], and furthermore all proteins hitherto examined have given gel films on compression. The behaviour found here is typical of mixed films of protein and fat, and further examination showed that considerable amounts of lipin could be extracted from the protein by means of alcohol and ether. When this had been done, the protein could no longer be spread on phosphate buffer (*p*H 7·0) from borax solution. An *N*/20 NaOH solution was therefore diluted with 20 vol. of 75 % ethyl alcohol to make an approximately 0·01 % solution, from which films could readily be spread. The limiting surface concentration at which the pressure was first measurable was about $1{\cdot}0 \times 10^{-7}$ g./cm.^{2} and the pressure rose to 10 dynes/cm. at $1{\cdot}8 \times 10^{-7}$ g./cm.^{2}, while gelation was observed at a pressure of 2 dynes/cm. These observations bring the latex protein into line with proteins previously studied.

Since both the lipin and protein had been extracted from the latex by what was essentially an aqueous solvent it was thought that the lipin might contain phosphatide. Analysis showed, however, that it contained no more than a trace of P and that it consisted largely of glyceride. In one particular experiment a crude product gave 2·9 g. lipin and 1·50 g. of protein. The lipin gave the following analysis:

N	0·07 %
P	Trace
Glycerides	91·65 % (fatty acids 87·7 %)
Unsaponifiable matter	7·14 % (81·4 % precipitated as digitonide)
Total recovered	98·79 %

Homogeneity of the various samples of protein

As stated above the yield of protein varied widely, on occasions being as high as 1·9 g. per kg. of dried latex; and in all 11·36 g. (after thorough extraction with alcohol and ether) were obtained from 9 kg. of dried latex. In view of the large variation in yield it was necessary to check the homogeneity of the products obtained from the different samples of dried latex. Lugg [1937; 1938] states that the tyrosine content of a protein may be estimated with a fair degree of accuracy (2 %), and estimations of this amino-acid were accordingly carried

out on the first sample of protein (0·5 g.) and on the bulk sample (11·36 g.) with the following results:

	Total wt. (g.)	Total N (%)	Tyrosine (%)	Tryptophan (%)
Batch 1	0·50	14·9	6·40	1·25
Bulk	11·36	15·0	6·40	1·35

These values suggest that the various samples of protein were of the same composition.

Analysis of the protein

The protein was analysed for the nine more important amino-acids, viz. tyrosine, tryptophan, cystine, methionine, arginine, histidine, lysine, glutamic acid and aspartic acid, and also for amide-N by micro and semi-micro methods which have been repeatedly checked, using standard proteins, by workers in this laboratory.

Amide-N. The protein was hydrolysed for 3 hr. with 2*N* HCl, and the liberated ammonia estimated by (Kjeldahl) distillation into standard acid.

Tyrosine and tryptophan. Triplicate estimations were carried out using the colorimetric method of Lugg [1937; 1938].

Total S. The method used was the alkali-fusion recommended by Bailey [1937] and Lugg [1938].

Cystine and methionine. These were estimated by Baernstein's method as modified by Lugg [1938].

Basic amino-acids. (Single determination.) These were estimated by Block's [1934] method as modified by Tristram [1939].

Dicarboxylic and basic amino-acids. The modification of the original Foreman [1914] method used in this laboratory [cf. Chibnall *et al.* 1940] requires 20–50 g. of protein, but owing to lack of material it was necessary to carry out the estimation on no more than 4·0 g. of protein. Trial experiments carried out on casein showed that, using 2·5 g. of protein, approximately 90 % of the glutamic and aspartic acids were recovered as hydrochloride and copper salt respectively, and that arginine and lysine could be accurately estimated in the filtrate from the alcoholic precipitation of the calcium salts (Table 3).

Table 3. *Analysis of casein*

Method	Wt. protein g.	Glutamic acid %	Aspartic acid %
Macro (Foreman)	70	21·0	6·2
Micro modification	2·5	18·7	5·76
		19·1	5·76
	Percentage recovery	90·4	93·0
		Arginine %	Lysine %
Tristram [1939]	2·5	3·76	6·02
The same, applied to dicarboxylic acid filtrate	2·5	3·84	5·93
	Percentage recovery	*ca.* 100	

The technique was applied to the rubber protein and the values obtained for the dicarboxylic acids (Table 4) were given a correction which assumed that the recovery observed was the same as in the case of casein. The results of these analyses are given in Table 4; for the purpose of comparison, the limiting values for leaf proteins analysed by similar methods (from Chibnall [1939]) have been included.

Table 4. *Analysis of latex protein*

	Protein of *Hevea* latex: N of amino-acid in % total protein-N	Amino-acid as % protein (N = 17 %†)	Various leaf proteins Amino-acid as % protein (N = 17 %)
Ammonia	7·07	1·46	0·97– 1·17
Arginine	12·9*	6·90	7·1 – 8·2
	13·0		
Lysine	6·4*	5·68	4·6 – 6·2
	6·4		
Histidine	1·0	0·63	1·0 – 1·6
Glutamic acid	7·65	13·68	11·4 –14·0
Aspartic acid	6·5	10·5	7·9 – 8·8
Tyrosine	3·3	7·24	4·8 – 6·1
Tryptophan	1·2	1·49	2·1 – 2·4
Methionine	0·62	1·12	2·2 – 2·7
Cystine	0·70	1·02	1·4 – 2·2
Total accounted for	47·4	49·72	
% Total N in product analysed	14·95	—	12·8 –15·0
Total S	0·70		

* Estimation carried out on the filtrate from the Ca salts of the dicarboxylic acids.

† The N content of the latex protein has been taken as 17 % for the purpose of comparison with the leaf proteins [cf. Chibnall, 1939].

Discussion

The nine amino-acids estimated represent 47·4 % of the total N. Since great difficulty was experienced in the estimation of cystine and methionine the values for these amino-acids must be regarded as minimal only.

It can be seen from Table 4 that the protein has many points of resemblance with those of typical leaf proteins; this is particularly marked in the case of the diamino-acids and the dicarboxylic acids, and the similarity suggests that there may be some relationship between the proteins in the leaves and in the latex of *Hevea brasiliensis*. Samples of protein have now been isolated from the leaves of *H. brasiliensis* and it is hoped to make comparative analysis in the near future.

Midgeley *et al.* [1937] stated that the protein present in rubber did not contain cystine, tyrosine and glutamic acid. The protein analysed by the author, however, contained 12 % glutamic acid, 6·4 % tyrosine and at least 1·0 % cystine (calculated as amino-acids on the protein). It is realized, however, that the two proteins in question may not be identical, since it is not claimed that the protein isolated by the extraction of dried latex with borate buffer represents the whole of the protein in latex. Further investigation is accordingly in progress to find what relation the protein so far isolated bears to the whole protein(s) of latex. It has often been stated [de Vries, 1924] that latex contains two proteins and that on coagulation one of these (A) remains in the serum while the other (B) is believed to be adsorbed on the precipitated rubber. If this theory be correct then dried latex will contain both A and B, and borate buffer might be expected to extract A preferentially. Crepe rubber on the other hand should contain B only. Preparations of protein from both these products are now being made by the method of Midgeley so as to test this supposition by comparative amino-acid analysis.

Summary

A protein has been isolated from dried *Hevea* latex and analysed for amide-N, tyrosine, tryptophan, cystine, methionine, the basic amino-acids and the dicarboxylic acids.

The dicarboxylic acids were estimated in 4·0 g. protein; experiments on casein having shown that, under such conditions, approximately 90% of the glutamic acid and aspartic acid were recovered as crystalline derivatives. It was also found possible to estimate arginine and lysine in the filtrate from the calcium salts of the dicarboxylic acids.

The protein is, in many respects, similar to those found in leaves of other plants (Table 4).

Further work is in progress to investigate the relationship of the isolated protein to the total protein of latex.

The author wishes to thank Prof. A. C. Chibnall, who was responsible for directing the course of the research, and whose advice and criticism have proved invaluable, and Dr G. Gee for kindly carrying out the surface film measurements.

The above work has been carried out as part of the programme of fundamental research on rubber undertaken by the Board of the British Rubber Producers' Research Association.

REFERENCES

Adam (1938). Physics and Chemistry of Surfaces, p. 87. Oxford.
Bailey (1937). *Biochem. J.* **31**, 1396.
Belgrave (1925). *Malay. Agric. J.* **13**, 367.
Bishop (1927). *Malay. Agric. J.* **15**, 27.
Block (1934). *J. biol. Chem.* **105**, 457.
Bondy & Freundlich (1938). *C.R. Lab. Carlsberg*, **22**, 89.
Chibnall (1939). Protein Metabolism in the Plant. New Haven: Yale Univ. Press.
—— Rees, Williams & Boyland (1940). *Biochem. J.* **34**, 285.
De Vries (1924). *Arch. Rubbercult.* **8**, 264.
Foreman (1914). *Biochem. J.* **8**, 463.
Frank (1914). The Rubber Industry, p. 144. London.
Freundlich & Hauser (1925). *Kolloidzschr. Z.* **36**, and *Zsigmondy Festschrift*, **15**.
Lugg (1937). *Biochem. J.* **31**, 1422.
—— (1938). *Biochem. J.* **32**, 2114.
—— (1939). *Biochem. J.* **33**, 110.
Midgeley, Henne & Renoll (1937). *J. Amer. chem. Soc.* **59**, 2501.
Miller (1935). *Biochem. J.* **29**, 2344.
Tristram (1939). *Biochem. J.* **33**, 1271.
Weber (1903). *Ber. dtsch. chem. Ges.* **36**, 3108.

44. THE USE OF A SYNTHETIC MEDIUM IN THE ISOLATION OF THE SOMATIC ANTIGENS OF *BACT. TYPHI-MURIUM* AND *BACT. TYPHOSUM*

By GEORGE GROSVENOR FREEMAN,
SYDNEY WILLIAM CHALLINOR
and JOYCE WILSON

From the Departments of Biochemistry and Bacteriology, London School of Hygiene and Tropical Medicine, University of London

(*Received 15 January 1940*)

Since the original description by Boivin & Mesrobeanu [1933] and by Raistrick & Topley [1934] of the isolation from organisms of the *Salmonella* group of non-protein chemical fractions possessing both toxicity and immunizing potency, other workers, and particularly Morgan [1937, 1, 2], have recorded work along similar lines. Three methods have been described by which such fractions can be isolated from the bacterial cell (1) by extraction with trichloroacetic acid [Boivin & Mesrobeanu], (2) by tryptic digestion [Raistrick & Topley], and (3) by extraction with diethyleneglycol. In another paper our colleague, Dr J. Walker, describes a fourth method which he has devised.

The work described in the present communication leaves little doubt that the fractions isolated by these different methods from any one organism are essentially the same. The work of the authors mentioned above indicates that the antigenic fractions consist essentially of a polysaccharide linked to a compound containing nitrogen and phosphorus, probably of a phospholipin nature. Their toxic and immunizing properties are closely similar, though there are minor differences [Henderson & Morgan, 1938] which indicate that the antigenic complex is probably affected, at any rate in its immunological properties, during the process of extraction. It is, however, extremely doubtful whether any one of the fractions yet prepared has been obtained in a state of chemical purity. The studies recorded in this paper, which are a continuation of those reported by Raistrick & Topley [1934], describe attempts to eliminate impurities derived from the starting material, or from the trypsin used for the digestion of the bacterial cells.

In all experiments referred to above, the large amounts of bacterial cells required for extraction have been obtained by cultivation on nutrient agar in Roux bottles, or in trays or Petri dishes, the growth being removed by scraping or washing. Since agar contains a large amount of polysaccharide it is clear that contamination of the starting material with this substance is likely to lead to contamination of a final fraction which itself consists largely of a complex polysaccharide. This difficulty has been encountered by other workers [Miles & Pirie, 1939].

This particular source of contamination can be avoided by growing the bacterial cells in a liquid medium, but such a highly complex medium as nutrient broth contains many constituents which might be carried through as contaminants to the final product. This difficulty can best be avoided by growing the bacterial cells in a simple synthetic medium.

We have found that commercial samples of trypsin, including that used in earlier experiments, contain large amounts of polysaccharide, while none are entirely free. The degree to which contamination from this source can be avoided is considered below.

The method described by Raistrick & Topley [1934] has been further improved by (*a*) removal of partially degraded cells by more efficient centrifuging of the tryptic digests and by filtration through Chamberland candles (L_2) and by (*b*) removal of low-molecular tryptic digestion products by dialysis in a collodion sac.

Experimental

(1) *Preparation of bacterial bodies*

The three methods employed in the preparation of the bacterial bodies which formed the starting material for our extractions were as follows:

(*a*) *Growth on nutrient agar in Roux bottles, and washed off.* Slight modifications of the method described by Raistrick & Topley [1934] were introduced. Batches of 90–100 bottles were put up at one time. Each bottle was inoculated with 3 ml. of an 18 hr. broth culture, incubated for 24 hr. at 37° and washed off with 7 ml. of 50 % acetone in sterile tap water, glass beads being used to assist in dislodging the growth of organisms. The suspension of bacteria was transferred to wide-mouthed, glass-stoppered jars, and an equal volume of acetone was added. The jars were incubated for 3 days at 37°, and their contents stirred each day with sterile glass rods. On the 3rd and 4th days, each jar was tested for sterility, and kept in the incubator until proved sterile. The supernatant acetone was then removed, replaced by an equal volume of absolute alcohol, and a final sterility test was performed before the product was used for chemical fractionation. The bacterial bodies were then centrifuged down, washed twice with alcohol and twice with ether and finally dried *in vacuo* over conc. H_2SO_4.

(*b*) *Growth on nutrient agar in metal trays; scraped off.* This method follows closely that described by Morgan [1937, 1]. Enamel trays $12 \times 10 \times 2$ in., each containing 500 ml. nutrient agar and covered with aluminium lids lined with filter paper, were inoculated with 50 drops (i.e. 1 ml.) of an 18 hr. broth culture, and the inoculum spread evenly over the surface by means of glass spreaders. After 24 hr. incubation at 37°, the growth was scraped off and suspended in sterile tap water in a wide-mouthed, glass-stoppered jar containing glass beads. An equal volume of acetone was added, and the subsequent treatment and sterility tests were the same as those used in the Roux bottle method. The supernatant acetone was removed by centrifuging and the bacterial bodies twice washed with alcohol at the centrifuge; two similar washings were then made with ether and the bacterial bodies dried *in vacuo* to a white powder.

(*c*) *Growth in synthetic medium.* Fildes *et al.* [1933] showed that although *Bact. typhosum* will not ordinarily grow in a medium without tryptophan, it can be trained to synthesize tryptophan when ammonia is the sole source of nitrogen. Recently, Gladstone [1937] studied the antigenic structure of *Bact. typhosum* grown in a synthetic medium which contained no tryptophan, and showed that in the presence of glucose the organism retains its Vi antigen, and is O-inagglutinable.

We have used the ammonia medium described by Gladstone, and, translating into quantities sufficient for the preparation of 60 l., the constituents are as follows:

KH_2PO_4	...	...	225 g.
$(NH_4)_2SO_4$	...	...	25 g.
NH_4Cl	...	...	25 g.

These salts are dissolved in 24,000 ml. of distilled water, and 1400 ml. *N* NaOH are added, the volume being made up to 30,000 ml. with distilled water. The reaction is adjusted to *p*H 7·6 by the addition of *N* NaOH, 18,250 ml. distilled water are added, and the medium is put up in 965 ml. quantities in 2 l. flasks and autoclaved at 115° for 20 min. To each flask the following solutions are added:

M/6 $MgSO_4, 7H_2O$	10 ml.	Mixed and added in one operation.
M/2 Glucose	25 ml.	
M $NaHCO_3$	10 ml.	

The glucose was sterilized by steaming for 30 min., and the other two solutions by autoclaving at 115° for 20 min. It seems important that these solutions should be added not more than 24–48 hr. before the medium is to be used, as storing for a longer period leads to a reduction in the amount of growth. In practice they are added on the morning when inoculations are to be made.

Bact. typhi-murium grew readily in this medium, giving an opacity of $3000–4000 \times 10^6$ organisms per ml. in 24 hr. We are indebted to Dr Gladstone for supplying us with a Vi strain of *Bact. typhosum* Ty_2 which had been trained to grow in a medium containing no added tryptophan. Both organisms remained fully virulent for mice when grown in the ammonia medium, and, apart from the loss of flagellar antigen, remained antigenically unaltered.

The usual procedure adopted for inoculating the flasks was as follows. The stock strains were kept on agar slopes in the ice chest, and subcultured about once a week. A generous loopful of culture was inoculated into a tube containing 10 ml. of ammonia medium and incubated for 24 hr. at 37°. About 5 ml. of this culture were put into a flask containing 250–500 ml. of ammonia medium, and incubated for 24 hr. at 37°. The following day 5 ml. of this culture were added to each litre of medium, and again incubated for 24 hr. at 37°. 5 ml. of 50 % formalin in sterile tap water were then added to each flask (making a final concentration of 0·25 %), and the flasks were stored at room temperature. After a further 24 hr., 0·5 ml. was removed from each and added to a tube containing 10 ml. of Hartley broth. These tubes were incubated for 3 days, examined daily for signs of growth, and plated on to agar and MacConkey medium on the 3rd day.

Recent experience has shown that satisfactory growth is obtained by seeding from the stock culture on the agar slope directly into the flask which is to be used for the mass inoculum, thus cutting out the preliminary growth in a tube of ammonia medium. The bacterial bodies were recovered from aqueous suspension in the culture medium by centrifuging in a De Laval Industrial Separator. The moist paste of organisms thus obtained was scraped from the bowl of the centrifuge, washed twice with alcohol, twice with ether and dried *in vacuo*.

(2) *Tryptic digestion of bacterial cells*

The technique of digestion of the dried bacterial bodies was based on the method of Raistrick & Topley [1934]. The organisms were submitted to digestion in batches of 10 g. The digestion mixture consisted of:

Dried bacterial bodies	10 g.
Sterile distilled water	2000 ml.
Trypsin (Fairchild Bros. and Foster, New York) ...	0·2 g.
Toluene	100 ml.

Half the trypsin was added at the beginning of digestion and the other half after 2·5 days. The digestion proceeded at 37°, *p*H 8·2–8·5 for 5 days. At the

outset sufficient alkali, usually about 4–5 ml. *N* NaOH, was added to bring the reaction to *p*H 8·2–8·5. The *p*H was determined approximately by means of a colorimetric method (B.D.H. comparator) and checked electrometrically, using a standard buffer and the "double hydrogen electrode". It was found that the *p*H tended to fall progressively during digestion and alkali was added when necessary to maintain the reaction at *p*H 8·2–8·5. Most of the alkali was required during the early rapid stage of digestion and the *p*H became fairly constant after 2–3 days. A typical digestion of *Bact. typhi-murium* bodies grown on synthetic medium required the addition of a total of about 18 ml. *N* NaOH. The course of the tryptic digestion has been followed by determination by the Van Slyke method of the amino-N liberated. Table 1 and Fig. 1 show

Table 1. *Rate of increase of amino-N during tryptic digestion of* Bact. typhi-murium *bodies*

Time hr.	Amino-N per ml. mg.	Total N per ml. mg.	Amino-N % of total N
0	—	0·609	—
1	0·055	0·609	9·0
3	0·073	0·609	12·0
8·5	0·102	0·609	16·8
23	0·147	0·609	24·2
30	0·151	0·609	24·8
48	0·165	0·609	27·1
60	0·172	0·609	28·2
72	0·196	0·609	32·2
77	0·205	0·609	33·6
82	0·209	0·609	34·3
96	0·209	0·609	34·3
102	0·215	0·609	35·3
120	0·212	0·609	34·8
456	0·223	0·609	36·6

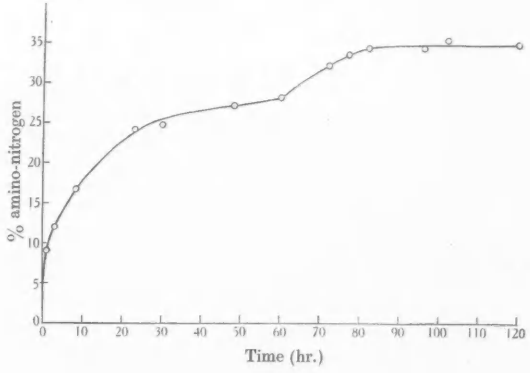


Fig. 1.

that there is an initial rapid increase of amino-N during the first 24–36 hr. followed by a steady rise during the remainder of the digestion period. The addition of more trypsin at the 60 hr. stage produces a slight increment of amino-acid liberation. In this experiment the amino-acid content had reached 34·8% amino-N after 5 days and 36·6% amino-N after 19 days; thus there is little further enzymic hydrolysis after the first 5 days.

After 5 days' digestion the very opalescent creamy-grey coloured liquid was neutralized to *p*H 7·0 by addition of *N* HCl, evaporated *in vacuo* at low temperature to about half volume, and clarified to an almost water-clear solution by passage through a De Laval Industrial Separator. The insoluble residue was washed from the centrifuge with alcohol, and after further washing with alcohol and ether was dried *in vacuo* to a cream-coloured powder ("undigested residue") which constituted about 17 % of the bacterial bodies (*Bact. typhi-murium* grown in synthetic medium).

The cleared centrifugate was passed through Chamberland L_2 candles, giving a very slightly opalescent filtrate which was concentrated *in vacuo* to about 100 ml. volume and dialysed in collodion sacs at room temperature, first against running tap water for 18 hr., and then against frequent changes of distilled water for a further 24 hr. During dialysis there was a 100–200 % increase in volume and the solution became opalescent. The dialysed liquid was concentrated *in vacuo* to 25–50 ml. volume and centrifuged on an angle centrifuge at 4000 r.p.m. for several hours. The residue was dried down to a white powder ("dialysis precipitate"), and constituted 0·2 % of the bacterial bodies (*Bact. typhi-murium* grown in synthetic medium). At this stage the solution gives an increased opalescence on addition of acetic acid to 0·1–0·2 *N* concentration and deposits a precipitate on centrifuging ("acetic acid precipitate"). This procedure of removal of an "acetic acid precipitate" has been adopted in the preparation of certain of the antigenic fractions. In general, however, the cleared supernatant solution was treated with alcohol to 68 % concentration by weight to precipitate a fraction, known as "tryptic digest F 68", as a fine white powder. This was washed twice with alcohol, twice with ether and dried *in vacuo* over phosphorus pentoxide.

The possibility of trypsin or impurities therefrom being present in the final antigenic fraction has already been mentioned. Samples of trypsin were obtained from several sources and their properties determined. This investigation included determination of (*a*) tryptic activity, (*b*) polysaccharide content and (*c*) amount and nature of the fraction precipitated from aqueous solution by alcohol under the conditions used in the bacterial digestions.

As a result of these experiments it was found that Fairchild Bros. and Foster trypsin had approximately five times as great an activity as a sample of trypsin used in the preliminary experiments. The activities were compared by the formaldehyde method using caseinogen as substrate. Fairchild Bros. and Foster trypsin gave on analysis 9·9 % N and 10·6 % reducing sugars on hydrolysis in a sealed tube for 2 hr. at 100° with 2 *N* HCl (Hagedorn & Jensen's method) as compared with 5·6 % N and 44 % reducing sugars for the trypsin used in the early experiments. In a control experiment under conditions precisely similar to those used in the preparation of "tryptic digest F 68", 1·20 g. of this trypsin were extracted with 12 l. of water for 5 days at *p*H 8·2–8·5, and 37°, and the extract centrifuged, filtered through a Chamberland L_2 candle, dialysed and treated with alcohol to 68 % concentration by weight; this gave 0·07 g. "trypsin F 68", i.e. 5·8 % of the original trypsin; the fraction gave on analysis 6·7 % N, and 23 % reducing sugars on hydrolysis. (The sample of trypsin used in preliminary experiments gave, under similar conditions, 57 % "trypsin F 68", which gave on analysis 1·2 % N and 50 % reducing sugars on hydrolysis under the conditions stated above.) Assuming the yield of antigen to be 5 %, which is an average figure from our results, the "trypsin" content of the product cannot exceed 4 % of the total.

In some recent experiments an attempt has been made to avoid all possibility

of contamination with impurities from commercial samples of trypsin by digesting bacteria with purified trypsin prepared according to Northrop & Kunitz [1932] in their preparation of crystalline trypsin. So far, we have found it impossible to degrade the bacterial cells with the purified enzyme to the same extent as with the commercial samples, and this problem is being further investigated.

(3) *Preparation of antigenic fraction by Boivin's method*

The method depends on extraction of bacterial bodies with ice-cold trichloroacetic acid and is based on the original method of Boivin & Mesrobeanu [1933] and Boivin *et al.* [1933, 1, 2; 1934]. The dried bacterial bodies were dispersed in five times their weight of water by grinding in a mortar and rapid mechanical shaking for 30 min. The suspension was cooled to 0° and an equal volume of ice-cold 0·2 *N* trichloroacetic acid added, the mixture being well shaken and kept at 0° for 3 hr. with occasional shaking. At the end of this period the very opalescent suspension was rapidly freed from the bulk of unextracted bacterial bodies by passage through a De Laval Industrial Separator, and the slightly opalescent centrifugate dialysed in collodion sacs at room temperature first against running tap water for 18 hr. and then against frequent changes of distilled water for a further 24 hr. After dialysis the trichloroacetic acid extract was again centrifuged through a De Laval Industrial Separator and the centrifugate passed through a Chamberland L_2 candle. The filtrates, which were usually still fairly opalescent, were concentrated *in vacuo* at low temperature and the specific bacterial substances precipitated by the addition of ethyl alcohol to 68% (by weight) concentration. The fraction thus precipitated was washed with alcohol and ether and dried *in vacuo* over phosphorus pentoxide to a white powder: "Boivin antigen F 68".

(4) *Preparation of antigenic fraction by extraction with ethyleneglycol or diethyleneglycol*

The method of extraction of bacterial bodies with ethyleneglycol and with diethyleneglycol used by Morgan [1937, 1, 2] for *B. dysenteriae* (Shiga) and Henderson & Morgan [1938] for strains of *Bact. typhosum* has been used in the preparation of antigenic fractions from *Bact. typhi-murium* and strains of *Bact. typhosum*. In our diethyleneglycol extractions we have used the distillate (B.P. 135–142° 10 mm.) of a commercial specimen of the solvent (B.D.H. "Technical"). The dried organisms (50 g.) were suspended in 500 ml. of the solvent at 37° by vigorous shaking in a stoppered bottle. A sample (0·5 ml.) of the suspension was removed, diluted with 4·5 ml. water and the *p*H determined electrometrically; this should be between 6·5 and 7·0. After 2 hr. at 37° the suspension was mechanically shaken for an hour and then kept at room temperature for 24 hr. During this period the suspension was frequently shaken by hand and finally mechanically shaken for an hour and kept for a further period of 24 hr. At the end of this time the bacterial suspension was freed from the bulk of suspended material by centrifuging in a De Laval Industrial Separator (or when smaller quantities of organisms were extracted the extract was clarified by centrifuging at 15,000 r.p.m. for 30 min.). The supernatant fluid was freed from any residual bacterial particles by filtration through a British Berkefeld filter candle followed by filtration through a Chamberland L_2 candle. The antigenic material was then obtained in aqueous solution free from the organic solvent by dialysis. During dialysis the slightly opalescent solution of the antigen in the organic solvent became strongly opalescent. At this stage the

antigen was isolated in a solid form by concentration of the solution *in vacuo* at low temperature and the addition of ethyl alcohol to 68% (by weight) concentration; the precipitate thus obtained was washed with alcohol and ether and dried *in vacuo* over phosphorus pentoxide.

The antigenic material soluble in the organic solvent is not always completely removed from the bacterial bodies by a single extraction. Where necessary, further extractions have been made in order to obtain a better yield; in these cases the number of extractions made is indicated in the relevant tables of results. These products have been labelled "ethyleneglycol or diethyleneglycol F 68".

The yield obtained by diethyleneglycol extraction of *Bact. typhosum* strain Ty_2 grown in synthetic medium (see Table 8) is considerably lower than that obtained by Henderson & Morgan [1938] and by Morgan (private communication), viz. 5% as a total of three extractions. In a series of subsidiary experiments the technique described above has been modified in attempts to improve the yield. These have included the use of only one bacterial filter, i.e. filtration of the diethyleneglycol extract through a Chamberland L_2 candle instead of filtration through a British Berkefeld filter candle followed by filtration through a Chamberland L_2 candle. This gave no significant increase in yield. When, however, cellophane membranes were used for dialysis instead of collodion sacs the yield was increased to 0·35%. Further experiments are proceeding with a view to determining the factors responsible for the differences between the yields we have obtained and those recorded by Morgan.

Contamination of antigenic fractions with polysaccharide material derived from agar or trypsin

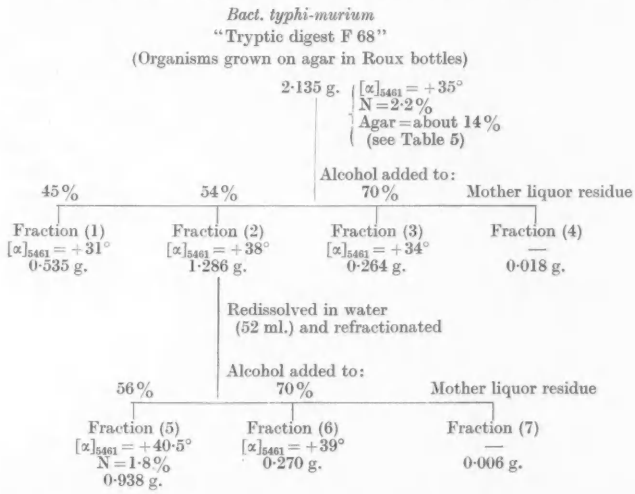
An appreciable amount of polysaccharide material can be removed from agar-grown organisms by aqueous washing. Thus 1·0 g. *Bact. typhi-murium* dried bodies, grown on agar medium in Roux bottles and washed off and killed with 50% acetone, was extracted three times with 100 ml. water and the extract centrifuged to remove bacterial bodies. The three extracts contained 0·122 g. (i.e. 12%), 0·039 g. (4%) and a trace respectively of solid material. The extracted material gave on analysis 33·2% reducing sugar on hydrolysis with 2*N* HCl for 2 hr. at 100° in a sealed tube (Hagedorn & Jensen method), 8·9% N and 8·9% non-volatile ash (the N and reducing sugar values are calculated on an ash-free basis). Aqueous washing of the bacterial cells, as a routine procedure to eliminate agar, was not considered satisfactory owing to the difficulties of eliminating the last traces of the soluble constituents by this method and the possibility of its removing part of the antigenic components of the cell.

Alcoholic or acetone fractionation of the antigenic fractions themselves was also tried, but it was found that these precipitating reagents behaved non-specifically in their precipitating actions on agar polysaccharides and the bacterial polysaccharides.

In the following experiment *Bact. typhi-murium* "tryptic digest F 68" from organisms grown on agar medium in Roux bottles was fractionated with alcohol and the fractions examined by determination of their specific optical rotations. Although this preparation contained about 14% of agar (see Table 5) there was no evidence of any laevorotatory compounds in any of the fractions obtained. (Agar yields a polysaccharide on alcoholic precipitation, $[\alpha]_{5461} = -35°$, see Table 9.) 2·1 g. of the antigenic fraction were dissolved in 70 ml. water and alcohol gradually added until a slight precipitate was obtained. This precipitate was then removed by centrifuging, washed twice with absolute alcohol, twice

with ether and dried *in vacuo* over phosphorus pentoxide. More alcohol was then added to the alcoholic supernatant solution to produce further precipitation and the procedure repeated in 3–4 stages until the final alcoholic supernatant solution was evaporated to dryness. The largest of the fractions was then again refractionated as shown in Table 2; the results in this table are uncorrected for ash.

Table 2. *Alcoholic fractionation of* Bact. typhi-murium *"tryptic digest F 68" from organisms grown on agar medium in Roux bottles*



Pirie [1936] describes methods for the detection and rough estimation of agar in biological materials. Use of these methods confirmed the presence of agar in antigenic fractions prepared from organisms grown on an agar medium in Roux bottles, but was of little value in determining its amount.

We have therefore adopted methods for roughly determining the extent of agar contamination of the dried bacterial bodies from various sources, depending on the galactose content of agar. It was found (*vide infra*) that bacterial bodies grown on a medium containing only mineral salts and glucose contained much less galactose than bodies grown on agar medium. These methods depend (*a*) on the oxidation of galactose to mucic acid, and (*b*) on the hydrolysis of the agar polysaccharides and precipitation of the galactose thus liberated as the methylphenylhydrazone.

(*a*) The oxidations were carried out according to Kent & Tollens [1885], who treated the galactose-containing substance on the boiling water bath with 10–12 times its weight of HNO_3 (sp. gr. 1·15), and evaporated to about $\frac{1}{6}$–$\frac{1}{8}$ the original volume. After cooling, $\frac{1}{6}$–$\frac{1}{8}$ of the original volume of cold water was added and the mixture well stirred to initiate crystallization. After 2–3 days' crystallization in the cold the mucic acid was collected on a small filter paper, washed with a few ml. of ice-cold water and dried at 100°. Under these conditions the oxidation of bacterial products was found to be complete in 1–2 hr. with 2 g. quantities of the substance. Mucic acid has also been obtained from the same

bacterial products by means of a shorter oxidation with conc. HNO_3. About 2 g. of the substance were boiled with 5–10 ml. of conc. HNO_3 for about 10 min. The mucic acid which crystallized on cooling and vigorous stirring was filtered off on a small filter paper, washed with a few ml. of ice-cold water and dried to constant weight at 100°. The results obtained with various samples of dried bacterial bodies and antigenic fractions are given in Table 3, (i) by Kent & Tollens method and (ii) by the short oxidation method. Melting points of the products are quoted and refer to the mucic acid as obtained without recrystallization. The yields of galactose stated in the final column are calculated on the basis of a 78% yield of mucic acid from pure galactose. Kent & Tollens [1885] also obtained a yield of about 78% mucic acid from galactose.

Table 3. *Yield of mucic acid on nitric acid oxidation of dried bacterial bodies and antigenic fractions*

Substance	Wt. oxidized g.	Yield of mucic acid g.	M.P. of mucic acid	Galactose %
Galactose	(i) 2·0	1·558	217°	100
Agar (Bacto-agar)	(i) 2·0	0·286	208°	18·3
	(ii) 2·0	0·360	212°	23·1
B.D.H. trypsin	(i) 2·0	0·273	214°	17·6
	(ii) 1·7	0·225	211°	17·0
Fairchild Bros. and Foster trypsin	(i) 2·0	0	—	0
Bact. typhosum (R.R.) F 68 (organisms grown on agar medium in Roux bottles—digested with B.D.H. trypsin)	(i) 0·5	0·081	214°	20·8
Bact. typhi-murium dried bodies grown on synthetic medium	(i) 2·0	0	—	0
Bact. typhosum Ty_2 dried bodies grown on synthetic medium	(i) 2·0	0	—	0
Bact. typhi-murium. Tryptic digest F 68. Organisms grown on synthetic medium digested with Fairchild Bros. and Foster trypsin	(i) 0·5	0	—	0

B.D.H. trypsin is the same sample as was used in the Raistrick & Topley [1934] work.

(*b*) Neuberg [1907] isolated galactose quantitatively, as the methylphenylhydrazone, from emulsin hydrolysates of raffinose. This method was subsequently used by Lüdtke [1929] and Neuberg & Schwietzer [1937] for the determination of galactose in agar and various polysaccharide fractions derived from it. Our procedure has been based on the methods of these authors; the bacterial bodies (2 g.) and antigenic fractions (0·5 g.) were hydrolysed on the boiling water bath for 3·5 hr. with 100 ml. *N* H_2SO_4.

Preliminary hydrolytic experiments with bacterial bodies showed that the acid hydrolysis was at a maximum after 3·5 hr. The hydrolysates were freed from any insoluble material by centrifuging and basic amino-acids and other protein degradation products were removed from the solutions by the addition of an excess (10 ml.) of 60% phosphotungstic acid in 5% H_2SO_4. The mixture was kept overnight at 0° and the phosphotungstic acid precipitate removed by centrifuging and washed with 2% phosphotungstic acid in 5% H_2SO_4. Excess

phosphotungstic acid and H_2SO_4 were removed by the addition of a very slight excess of hot saturated baryta to the acid hydrolysate, cooled in ice. The precipitated Ba salts were removed by centrifuging and washed with dilute baryta and the combined supernatant solution and washings made just acid by addition of $2N$ H_2SO_4. The excess acid was removed by addition of a slight excess of pure $BaCO_3$ and the precipitates removed by filtration. The clear neutral and almost colourless filtrate was evaporated *in vacuo* at low temperature to 10 ml. volume. A few drops of dilute acetic acid were added during the evaporation to keep the solution just acid to litmus. 10 ml. absolute ethyl alcohol were then added and the mixture kept at 0° overnight; any precipitate which had separated out was removed by centrifuging. The filtrate was treated with 0·4 g. of methylphenylhydrazine (i.e. at least twice the calculated quantity) and 0·1 ml. 50 % aqueous acetic acid, kept at 35–38° for 6–7 hr. with occasional gentle shaking and then in the ice chest for about 15 hr. At the end of this period the hydrazone obtained was collected on a tared Gooch crucible, washed with 10 ml. ice-cold ethyl alcohol and dried to constant weight *in vacuo* over phosphorus pentoxide. Where necessary the product was recrystallized from hot absolute alcohol until pure. Table 4 shows the results obtained for various bacterial products and a control experiment on pure galactose.

Table 4. *Determination of galactose in dried bacterial bodies and antigenic fractions*

Substance	Wt. g.	Yield of galactose methylphenyl-hydrazone g.	Nature of methylphenylhydrazone	Galactose %
Galactose	0·2239	0·3353	Colourless crystalline plates, M.P. 187°	95·0
Bact. typhi-murium dried bodies grown in synthetic medium	2·01	0·0234	Colourless micro-crystalline plates, M.P. 184°	0·74
Bact. typhi-murium dried bodies grown on agar medium in Roux bottles	1·809	0·0367	Colourless micro-crystalline plates, M.P. 185°	1·29
Bact. typhosum Ty_2 dried bodies grown in synthetic medium	1·928	0·0195	Colourless micro-crystalline plates, M.P. 186°	0·64
Bact. typhi-murium "tryptic digest F 68" from organisms grown on agar medium in Roux bottles, digested with Fairchild trypsin	0·5739	0·0552	Colourless micro-crystalline plates, M.P. 183°	6·10
Bact. typhi-murium "tryptic digest F 68" from organisms grown in synthetic medium, digested with Fairchild trypsin	0·4908	0·0204	Colourless micro-crystalline plates, M.P. 184°	2·64
Bact. typhosum Ty_2 "tryptic digest F 68" from organisms grown in synthetic medium, digested with Fairchild trypsin	0·4449	0·0080	Colourless micro-crystalline plates, M.P. 185°	1·14

Comparing Tables 3 and 4 it is found that the bacterial bodies from a synthetic medium and antigenic fractions prepared from them yield small though significant amounts of galactose on hydrolysis, which are detectable by the

sensitive methylphenylhydrazone method but cannot be detected by the cruder method of oxidative conversion of galactose into mucic acid.

If the assumption be made that the organisms synthesize the same amounts of galactose when grown on an agar medium as in the synthetic medium the above results quoted in Table 4 may be used to obtain a measure of the extent of agar contamination of bacterial bodies grown on agar media and of the antigenic fractions prepared from them.

Various workers have obtained figures of 25–30% as the galactose content of agar (Neuberg & Schwietzer [1937], Lüdtke [1929] and Pirie [1936]) and we have obtained 23% by the mucic acid method from unpurified agar. On the basis of a 25% yield of galactose from agar, the following results are obtained for agar contamination of the bacterial bodies and antigenic fractions investigated.

Table 5

Substance	Organisms grown in synthetic medium: galactose %	Organisms grown on agar medium in Roux bottles: galactose %	Difference in galactose content %	Agar contamination %
Bact. typhi-murium dried bodies	0·74	1·29	0·55	2·2
Bact. typhi-murium "tryptic digest F 68"	2·64	6·10	3·46	13·8

In an attempt to reduce the agar contamination of the dried bacterial bodies grown on Roux bottles, two other methods of growth were investigated as described above. The dried bodies, prepared as already described from the three sources, have been submitted to chemical analysis and the results for N content and reducing sugar values, calculated on an ash-free basis, are given in Table 6.

Table 6. *Analysis of dried bacterial bodies*

	Bact. typhi-murium			*Bact. typhosum* R.R.	*Bact. typhosum* Ty_2	
	Grown on agar medium in Roux bottles	Scraped from agar medium on trays	Grown in synthetic medium	Grown on agar medium in Roux bottles	Grown on agar medium in Roux bottles	Grown in synthetic medium
Yield	17·5 g. per 100 Roux bottles	73 g. per 100 trays	17·5–20 g. per 100 l.	15 g. per 100 Roux bottles	13 g. per 100 Roux bottles	13 g. per 100 l.
N %	13·7	14·8	13·4	13·4	13·0	13·4
Non-volatile ash %	8·2	3·8	6·5	8·6	9·5	7·4
Reducing sugars on hydrolysis for 2 hr. at 100° with 2 ml. 2*N* HCl in a sealed tube (Hagedorn & Jensen) %	14·1	13·9	16·4	14·5	14·6	14·9

Properties of the antigenic fractions

Various analytical results and the results of toxicity experiments in mice on antigenic fractions prepared from organisms grown by the methods 1 (*a*), (*b*) and (*c*) described above and fractionated according to the techniques described under (2), (3) and (4) are summarized in Tables 7 and 8.

Samples of antigens and bacterial bodies used in the various analytical determinations have been dried in small weighing tubes at 10–20 mm. and 61°,

with passage of a slow stream of dry air, for a standard time of 45 min. Just sufficient of the sample for the determination in question, e.g. 5–10 mg. for N determination, was dried in this way. The N analyses throughout the work have been made by the micro-Kjeldahl technique of Pregl. Ash determinations were made by ashing 5–10 mg. of substance in a platinum boat in a micro-muffle furnace. The reducing sugar determinations were carried out according to the method of Hagedorn & Jensen; 10 mg. quantities of substance were hydrolysed by heating on the boiling water bath with 2 ml. of 2*N* HCl for 2 hr. in a sealed tube; the hydrolysates were neutralized with 4*N* NaOH, diluted to 10 ml. and determinations made on 1 ml. samples. In the determinations of specific rotation about 100 mg. of substance were dissolved in 10 ml. water (a few micro-determinations were also made using 10 mg. of substance dissolved in 1 ml. water). The antigenic fractions dissolve to form slightly opalescent solutions and there is a tendency towards reversion to an insoluble form on drying which renders redispersion more difficult. Whenever it was impossible to determine the specific rotation on the solution at this stage, it was submitted to high speed centrifuging (usually 15,000 r.p.m. for 30 min.) until sufficiently clear for the determination to be made. Readings were made on the cleared supernatant solution in a 1 dm. tube using the mercury green line λ_{5461} Å. When there was separation of an appreciable quantity of insoluble residue on centrifuging, this was washed twice with alcohol, twice with ether, dried over phosphorus pentoxide and weighed. The weight of insoluble residue was deducted from the original weight of the sample in calculating the concentration of the rotation solution. The N contents, reducing sugar values and specific rotations are calculated on an ash-free basis.

The determinations of toxicity were made by the intraperitoneal injection into mice of 0·5 ml. of solutions containing falling amounts of the fraction under test. Each dose tested was injected into five mice, and the dose injected was halved in successive groups of mice, the range of dose employed in most tests being from 4 to 0·125 mg. with the various fractions obtained from *Bact. typhimurium*, and from 8 to 0·25 or 0·125 mg. with the fractions obtained from *Bact. typhosum*. For the method employed in calculating the average lethal dose, and its standard error, we are indebted to our colleague, Dr J. O. Irwin, who has recorded elsewhere [Irwin & Cheeseman, 1939] the statistical method itself and the results obtained in a series of parallel tests with some of our material. The standard errors have not been included in Tables 7 and 8, but it may be stated that, on the customary assumptions, the average lethal dose recorded must be regarded as subject to an experimental error of approximately +100 or −50%, i.e. the true average lethal dose is unlikely to be more than twice, or less than half, the figure given.

Where many determinations have been made on similar preparations, as has been the case with the tryptic digest F 68 fractions from growth on Roux bottles, or in synthetic medium, the range given for the average lethal dose may be accepted with considerable confidence. Where only a single determination has been made the figure recorded should be treated with equal reserve. In a few instances in which an unusually wide range in A.L.D. is shown, as for instance, in the second tryptic digest fraction from the R.R. strain of *Bact. typhosum* (Table 8), a long period elapsed between successive tests on the same fraction. Since the higher A.L.D. values were obtained in the later tests it is possible that the toxicity of the fractions tend to decrease on storage, though in many samples we have found that the A.L.D. value remains relatively constant over periods of a year or more.

Table 7. *Properties of antigenic fractions prepared from* Bact. typhi-murium

Outline of method of preparation	Grown on agar medium in Roux bottles: "Tryptic digest F 68" I	"Boivin antigen F 68"	"Ethylene-glycol F 68"	Grown on agar medium and scraped off trays: "Tryptic digest F 68" V (*a*) (Organisms acetone-killed) Dialysis in cellulosic membrane	"Tryptic digest F 68" V (*b*) Dialysis in collodion membrane	"Tryptic digest F 68" VI (organisms acetone-killed)	"Boivin antigen F 68" (organisms formalin-killed)
Acetic acid conc. *N*	0·1	—	—	—	—	0·2	—
Yield of fraction %	8·4	7·2	5·8 (1st extraction)	2·9	3·5	—	2·2
N %	2·4	2·6	3·3	4·3	2·8	3·4	3·0
Non-volatile ash %	7·6	7·1	9·6	8·2	7·4	8·3	6·6
Reducing sugar value %	54	49	47	39	52	43	56
$[\alpha]_{5461}$	+38°	+43°	+34°	—	+90° (approx.)	+65° (approx.)	+91°
Average lethal dose for mice mg.	0·13–0·31	0·15–0·20	0·27–0·93	—	—	—	0·13

Outline of method of preparation	Grown in synthetic medium: "Tryptic digest F 68" VII	"Tryptic digest F 68" VIII	"Tryptic digest F 68" IX	"Tryptic digest F 68" XI	"Tryptic digest F 68" XII	"Tryptic digest F 68" XIII	"Boivin antigen F 68"	"Diethylene-glycol F 68" (1st and 2nd extractions)
Acetic acid conc. *N*	0·2	0·3	0	0	0	0	—	—
Yield of fraction %	3·9	4·4	5·9	6·5	6·0	—	3·5	0·5
N %	3·0	5·4	6·1	4·1	6·0	4·1	2·5	2·4
Non-volatile ash %	6·1	9·6	12·5	8·2	9·7	7·4	9·0	9·7
Reducing sugar value %	51	48	47	51	47	51	48	51
$[\alpha]_{5461}$	+75° (approx.)	+83°	+83°	+80°	+80°	+77°	+47°	+78°
Average lethal dose for mice mg.	0·13–0·27	—	—	—	—	—	0·47	0·61

Table 8. *Properties of antigenic fractions prepared from strains of* Bact. typhosum

Outline of method of preparation	Strain R.R. grown on agar medium in Roux bottles: "Tryptic digest F 68" I	"Tryptic digest F 68" II I after treatment with 0·1 *N* HOAc in the cold	"Ethylene-glycol F 68"	Strain Ty_2 grown on agar medium in Roux bottles "Tryptic digest F 68" I	Strain Ty_2 grown in synthetic medium: "Tryptic digest F 68" I	"Tryptic digest F 68" III	"Tryptic digest F 68" IV	"Boivin antigen F 68"	"Diethy-lene-glycol F 68"
Acetic acid conc. *N*	0	0·1	—	1·0	0	0	0	—	—
Yield of fraction %	14	12	7·2 (1st extraction)	7·7	5·2	5·1	3·4	3·6	0·06 (1st extraction)
N %	5·2	5·0	5·7	4·2	7·7	6·8	7·0	4·2	4·3
Non-volatile ash %	11·5	11·1	21·3	9·1	12·0	12·5	12·2	9·1	13·7
Reducing sugar value %	37	42	39	46	30	35	32	36	—
$[\alpha]_{5461}$	+38°	+42°	+45°	+81°	+112°	+93°	+107°	+90°	—
Average lethal dose for mice mg.	1·4	0·41–2·1	—	0·93	0·31	—	—	0·23	—

Discussion

The three main methods of preparation of antigenic fractions which have been used in this work have yielded, from comparable starting materials, products which show remarkable similarities in chemical and immunological properties. They are all complex, non-dialysable substances containing N and giving rise to reducing sugars on hydrolysis. The fractions are all water-soluble, giving solutions which are optically active (dextrorotatory). The solutions exhibit the property, characteristic of their high molecular complexity, of

partial reversion to an insoluble form on drying and storage. Although the fractions at this stage cannot be regarded as chemical entities, the similarity of chemical composition and of toxicity in mice between the substances prepared by very different methods, from the uncontaminated starting material—bacterial bodies grown in a synthetic medium—lends support to the view that we are now dealing with a product which consists predominantly of the antigenic complex of the bacterium.

In view of the high percentage of reducing sugars obtained on hydrolysis of the antigenic fractions and the probability that a polysaccharide complex may play an important part in the immunological specificity of the antigen, it has been considered worth while to investigate systematically the problem of agar contamination of the starting materials and the antigenic fractions. The colour tests devised by Pirie, whilst confirming the presence of agar derivatives in the bacterial bodies grown on an agar medium and antigenic fractions prepared from them, were of limited value in assessing the amount of agar present. It has been possible, however, to obtain an accurate estimate of the extent of contamination of bacterial bodies and antigenic fractions by the determination of their galactose content, as compared with that of similar substances obtained from agar-free sources. The extent of agar contamination present in the dried bacterial bodies grown on an agar medium in Roux bottles has been determined by this means and appears to be of the order of 2 % of the bacterial bodies. The results in Table 6 show that the organism grown in synthetic medium contained less N than bodies grown on agar and that the reducing sugars obtained on hydrolysis are correspondingly higher. This is probably associated with a change in the organisms occasioned by growth in a synthetic medium free from tryptophan. However, the immunological properties of the antigen obtained from bodies grown in this way do not appear to be materially affected.

Bact. typhi-murium *antigenic fractions*

Sample analyses of *Bact. typhi-murium* fractions prepared by three different methods from bacterial bodies grown both on agar media and in a synthetic medium are given in Table 7. In the case of the "tryptic digest F 68" from organisms grown in synthetic medium (antigenic solution untreated with acetic acid) four parallel preparations were made and the products analysed. The results are not strictly reproducible from batch to batch but the variations in the reducing sugar value (47–51 %) and the specific optical rotation $[\alpha]_{5461}$ +77–83° are relatively small, although there is a greater variation in the N contents (4·1–6·1 %). The same order of reproducibility is to be expected for the other fractions for which, in general, single analyses have been quoted.

Two fractions prepared from bacterial bodies, grown on nutrient agar in trays and scraped off, gave analytical results departing widely, particularly in N content, from all our other findings. The figures for these two samples are not included in Table 7. The cause of the discrepancy remains unknown.

The products prepared from organisms from an agar medium in Roux bottles by the three methods have N contents varying from 2·4 to 3·3 %, reducing sugar values 47–54 % and specific optical rotations, +34 to +43°, which present the same order of variation as the replicate batches quoted above. A similar argument holds for the fractions from organisms grown on agar medium and scraped off trays and for those fractions from organisms grown in the synthetic medium. The specific optical rotation (+47°) for the "Boivin antigen F 68" prepared from organisms grown in the synthetic medium differs widely from that obtained for the other fractions from this source; this may be

associated with differences of molecular complexity which would not affect the other analytical data.

The yields of the fractions prepared by the tryptic digestion method are higher than those prepared by the other methods.

Consideration of the average analytical figures for the products prepared by all three methods from organisms grown on the one hand on agar medium, and on the other on a synthetic medium, confirms the fact, established by the galactose estimations already described, that the antigenic fractions prepared from bacterial bodies grown in Roux bottles contain considerable amounts of "agar polysaccharides". The mean analytical figures may also be used to furnish a very rough indication of the extent of this contamination. Table 9 shows the average analytical figures for the antigenic fractions from the three main sources investigated, together with data for an agar polysaccharide fraction obtained by precipitating bacto-agar solution with alcohol to 68% concentration by weight, and calculated results for a hypothetical fraction consisting of 2 parts of antigenic fraction from organisms grown on the synthetic medium and 1 part "agar polysaccharide".

Table 9. *Mean analytical data of* Bact. typhi-murium *antigenic fractions*

	Method of growth of organism, from which fractions were prepared				
	Agar medium in Roux bottles	Agar medium in trays. Organisms scraped off	Growth in synthetic medium (*a*)	"Agar polysaccharide" fraction (*b*)	Hypothetical fraction 2 parts (*a*) + 1 part (*b*)
Yield %	7·1	2·9	4·4	—	6·8
N %	2·8	3·4	4·2	0·5–0·6	2·9
Reducing sugar value %	50	48	50	50	50
$[\alpha]_{5461}$	+38°	+82°	+76°	−35°	+39°

Comparing our analytical figures with the values recorded in earlier papers, we find that the results are of the same order although there are certain differences, particularly in the N contents. Boivin's antigen from *Bact. typhi-murium* contained 1·83–2·20% N and gave 40% of reducing sugars on hydrolysis [Boivin & Mesrobeanu, 1934] as compared with our values, for the product obtained by the same method, of 2·5% N and 48% of reducing sugars on hydrolysis. Topley *et al.* [1937] obtained an antigen from *Bact. typhi-murium* containing 4·2–4·8% N; in our tryptic digestion work the N content of the purest fractions has varied from 4·1 to 6·1%. Raistrick & Topley [1934] obtained 23·1% of reducing sugars on hydrolysis of their crude antigen F 68 and 37·8% from F 68/68; our tryptic digest antigens from organisms grown in the synthetic medium yield 47–51% of reducing sugars on hydrolysis. These figures might appear to conflict with the view that the products of the earlier workers contained "agar" and other impurities from the culture media, but it must be remembered that efficient removal of bacterial debris by centrifuging and filtration, and elimination of low-molecular breakdown products of the tryptic digestion by dialysis rather than alcoholic fractionation, have also modified the analytical data of the fractions.

Bact. typhosum *antigenic fractions*

The mean analytical data for the *Bact. typhosum* fractions are summarized in Table 10.

Table 10. *Mean analytical data of* Bact. typhosum *antigenic fractions*

	Method of growth of organism, from which fractions were prepared, etc.		
	Strain R.R. Roux bottle grown	Strain Ty_2 Roux bottle grown	Strain Ty_2 Synthetic medium. Tryptic digest fractions
Yield %	11·1	7·7	4·8
N %	5·3	4·2	7·2
Reducing sugar value %	39	46	32
$[\alpha]_{5461}$	+42°	+81°	+104°

Comparison of these averages with the results in Table 8 shows that, firstly, parallel products from the organisms grown in the synthetic medium yield closely reproducible analytical data. So far as the products from a Roux bottle source are concerned, strain R.R. has yielded fractions by the three different methods of preparation which are closely similar, and it is again clear, as in the case of the *Bact. typhi-murium* fractions, that these three methods give rise to essentially the same polysaccharide products from the bacterial bodies. (In our hands the yields by extraction with glycols have always been lower than those by other methods.)

From a comparison of the analytical data for strain Ty_2 fractions prepared, on the one hand, from organisms grown on an agar medium in Roux bottles and, on the other hand, from organisms from a synthetic medium (Table 10) it is clear that the former contains an appreciable quantity of contaminating agar polysaccharides.

The N content of our "diethyleneglycol F 68" from strain Ty_2 grown in synthetic medium (4·3 %) is much higher than that quoted by Henderson & Morgan [1938] of 2·25–2·4 % N with a similar O+Vi strain. The same is true for the corresponding "Boivin antigen F 68"—4·2 % N as compared with 2·5 % obtained by Boivin. Topley *et al.* [1937] obtained a tryptic digest antigen from strain R.R. which contained 4·8–5·3 % N, and from the H 901 strain, 4·9–5·1 % N.

The analytical results in columns 1 and 2 of Table 8, and also those of *Bact. typhi-murium* fractions derived from the synthetic medium (Table 7) indicate that removal of an acetic acid-insoluble fraction of the antigen does not greatly alter its nature. It has already been pointed out that the antigenic fractions are readily susceptible to mutation to an insoluble form and it appears that treatment with acetic acid accelerates this, rather than produces a sharp separation of a chemically distinct fraction. Use of acetic acid is therefore not recommended at this stage of the preparation of the fractions.

Reference to Table 3 will show that an early crude antigenic fraction from strain R.R. organisms grown on agar medium in Roux bottles and digested with B.D.H. trypsin yielded mucic acid equivalent to 20·8 % of galactose. This is due to the presence in the antigenic fraction of galactose-containing polysaccharides from the commercial trypsin as well as of agar.

Boivin & Mesrobeanu [1938] describe a method of separation of the O and Vi components of antigens from *Bact. typhosum* by means of precipitation of the Vi antigen with uranyl acetate. The precise chemical differences between these components have not yet been elucidated. We have preferred, therefore, at this stage to consider the antigenic complex from *Bact. typhosum*, strain Ty_2, as directly extracted by the methods described.

Comparison of antigenic fractions from Bact. typhi-murium *and* Bact. typhosum *strain* Ty_2 *grown in synthetic medium*

Both the antigenic fractions prepared from *Bact. typhi-murium* and *Bact. typhosum* strain Ty_2, organisms grown in synthetic medium and extracted by the three different methods, appear to belong to the same type of compound, i.e. a N-containing, non-dialysable water-soluble substance which yields reducing sugar on hydrolysis. They are both strongly dextrorotatory. The untreated antigenic fractions have no appreciable reducing power, and contain a negligible amount of amino-N. The fractions from the two organisms, though qualitatively similar, differ in their analytical data. Thus the fractions obtained from *Bact. typhosum* Ty_2 grown in synthetic medium all contain significantly greater quantities of N and have higher specific optical rotations than the corresponding fractions from *Bact. typhi-murium*. The reducing sugar values are however markedly lower.

SUMMARY

Methods of growth of bacterial bodies of *Bact. typhi-murium* and *Bact. typhosum* for the subsequent chemical extraction of antigenic fractions are described and have been critically examined. It has been found possible to obtain chemical fractions of these organisms free from contaminating substances, such as agar from the media, only when the organisms are grown in a liquid medium, preferably containing only inorganic salts and glucose.

Antigenic fractions have been prepared from these organisms by three methods: (*a*) a development of the tryptic digestion method of Raistrick & Topley; (*b*) Boivin's method of extraction with cold trichloroacetic acid and (*c*) extraction with organic solvents such as ethyleneglycol and diethyleneglycol [Morgan, 1937, 1], and the products have been compared by chemical analysis and the determination of toxicity in mice. The purest products obtained by these methods from organisms free from contaminants of the culture media show such a similarity of properties that it may be concluded that by any of these methods the essential antigenic complex of the bacteria may be obtained in a state approaching chemical homogeneity.

On the grounds of yield and suitability for use in extracting large quantities of bacterial bodies the tryptic digestion method and Boivin's method are recommended. Quantities of these products are being accumulated for further chemical fractionation.

A method for determining the galactose content of bacterial bodies and antigenic fractions has been elaborated and can be applied to the problem of the determination of the extent of agar contamination of bacterial fractions obtained from agar media.

These studies, and others in the same general field are, of necessity, extremely expensive. They involve the growth of relatively enormous masses of pathogenic bacteria, laborious and large-scale chemical manipulations, and the annual expenditure of many thousands of mice in the immunological assay of the various chemical fractions isolated. They have been rendered possible by grants received from many different sources: from the Leverhulme Trust, the Pilgrim Trust, Imperial Chemical Industries, Ltd. and from a fund raised by the late Sir Austen Chamberlain, to which many generous private donors have contributed. One of us (J. W.) has recived a research grant from the Medical Research Council, two of us (G. G. F. and S. W. C.) have held Grocers' Company Scholarships for Medical Research, and Miss C. Begg, whom we wish to thank

for much assistance in the preparation of bacterial bodies, was the holder of a Halley Stewart Research Fellowship. To all these we would express our thanks.

The authors wish to thank Profs. H. Raistrick and W. W. C. Topley for much advice and criticism, and Mr T. H. Anderson for help with the analytical work.

REFERENCES

Boivin & Mesrobeanu (1933). *C.R. Soc. Biol., Paris*, **112**, 76.
—— —— (1934). *C.R. Acad. Sci., Paris*, **198**, 2211.
—— —— (1938). *C.R. Soc. Biol., Paris*, **128**, 5.
—— —— & Mesrobeanu (1933, 1). *C.R. Soc. Biol., Paris*, **113**, 490.
—— —— —— (1933, 2). *C.R. Soc. Biol., Paris*, **114**, 307.
—— —— —— (1934). *C.R. Acad. Sci., Paris*, **198**, 2124.
—— —— —— (1935). *Arch. roum. Path. exp. Microbiol.* **8**, 45.
Fildes, Gladstone & Knight (1933). *Brit. J. exp. Path.* **14**, 189.
Gladstone (1937). *Brit. J. exp. Path.* **18**, 67.
Henderson & Morgan (1938). *Brit. J. exp. Path.* **19**, 82.
Irwin & Cheeseman (1939). *J. Hyg., Camb.*, **39**, 574.
Kent & Tollens (1885). *Liebigs Ann.* **227**, 221.
Lüdtke (1929). *Biochem. Z.* **212**, 419.
Mesrobeanu (1936). *Arch. roum. Path. exp. Microbiol.* **9**, 1.
Miles & Pirie (1939). *Brit. J. exp. Path.* **20**, 83.
Morgan (1937, 1). *Biochem. J.* **31**, 2003.
—— (1937, 2). *Chem. Ind. Rev.* **56**, 446.
Neuberg (1907). *Biochem. Z.* **3**, 519.
—— & Schwietzer (1937). *Mh. Chem.* **71**, 46.
Northrop & Kunitz (1932). *J. gen. Physiol.* **16**, 267, 295, 313, 323, 339.
Pirie (1936). *Brit. J. exp. Path.* **17**, 269.
Raistrick & Topley (1934). *Brit. J. exp. Path.* **15**, 113.
Topley, Raistrick, Wilson, Stacey, Challinor & Clark (1937). *Lancet*, **1**, 252.

45. A METHOD FOR THE ISOLATION OF TOXIC AND IMMUNIZING FRACTIONS FROM BACTERIA OF THE *SALMONELLA* GROUP

By JAMES WALKER

From the Dyson Perrins Laboratory, Oxford University

(*Received 15 January 1940*)

Toxic and antigenically active fractions consisting of polysaccharides linked to a component containing nitrogen, phosphorus and fatty acids, but devoid of demonstrable protein have been isolated from bacteria of the coli-typhoid group by three different methods; by extraction with $N/4$ trichloroacetic acid [Boivin *et al.* 1933, 1, 2], by tryptic digestion [Raistrick & Topley, 1934] and by extraction with diethyleneglycol [Morgan, 1937; Henderson & Morgan, 1938]. The products obtained by these three methods from any one bacterial species correspond closely in their chemical, toxic and antigenic properties, though minor differences have been recorded in their antigenic behaviours. It would seem that they are very nearly identical although the methods used in their preparation have differed so markedly. Since, however, tryptic digestion has been used for the isolation of the bound polysaccharide from egg albumin [Neuberger, 1938] that method would seem to come under suspicion from the point of view of providing an entirely native product when applied to bacteria. The extremely acid nature of Boivin's solvent has been commented upon by that author himself [Boivin *et al.* 1938] and, as Morgan [1937] has emphasized, it seems improbable that the immunological properties of labile antigens will remain unaltered on exposure for any appreciable length of time to degrees of acidity and alkalinity which are far removed from neutrality. It was deemed desirable, therefore, to look for another technically simple method which should (*a*) be operative at a neutral reaction, and (*b*) introduce no substance incapable of subsequent ready elimination; these conditions are fulfilled by Morgan's [1937] method which yields toxic and immunizing products, but his procedure is lengthy and recent work [Henderson, 1939] would seem to indicate that an intact product is not obtained.

Experiments were therefore instituted along the following lines. It is well known that aqueous urea solutions have the power of peptizing proteins [cf. Spiro, 1900; Burk & Greenberg, 1930] and on dilution or on dialysing away the urea again the protein is frequently precipitated spontaneously [cf. e.g. Anson & Mirsky, 1929–30]. It was hoped, therefore, that by using urea solutions it would be possible to peptize the bacterial cells in such a way as to liberate the cell contents and then, by suitable means, either by dialysis or by the use of a specific precipitant, to render the protein insoluble and provide in this way a solution of the carbohydrate-containing protein-free antigen, which could subsequently be isolated in the free state. These expectations were, however, not realized since practically no dissolution of the cells could be demonstrated within the range of urea concentrations used [cf. also Fuller, 1938]. On the other hand, it was found that the solutions used had the power of extracting from the bacteria carbohydrate-containing, possibly protein-free, toxic substances which conferred a definite degree of antibacterial immunity on mice.

In the present work the organisms have been grown largely on the chemically defined medium described in the preceding paper by Drs Freeman, Challinor and Wilson, who discuss critically possible sources of contamination of bacterial bodies with agar, etc., since it is only after the elimination of all possible sources of contamination of the bacterial cells, either with components of the media upon which they are grown [cf. Sordelli & Mayer, 1931; Morgan, 1936] or with reagents used in their manipulation [cf. Delafield & Smith, 1939], that a reliable picture of bacterial synthetic activity will become available.

Preliminary experiments were carried out with *Bact. typhi-murium* and *Bact. typhosum* (strain R.R.) grown on nutrient agar and from these organisms toxic and immunizing fractions were isolated. Subsequent work was carried out with *Bact. typhi-murium* and *Bact. typhosum* (strain Ty_2) grown on the above-mentioned synthetic medium and only toxicity tests were carried out on the products obtained for reasons of economy both in mice and in time. No claim is made that the products were homogeneous and fractionation will have to await the accumulation of larger amounts than have hitherto been available. The yields obtained are of an order comparable with those obtainable by the method of Boivin & Mesrobeanu [1935] and rather less than those obtainable by that of Raistrick & Topley (see preceding paper); as far as can be seen at present the only significant differences between the present preparations and the products obtained by the other methods lie in the slightly higher nitrogen and lower carbohydrate contents of the urea extraction products where comparative figures are available. The likelihood of urea having entered into chemical combination with the bacterial product is not considered great and, since no significant amounts of protein can be present, some other reason must underlie the higher nitrogen values found for the present fractions. Prolongation of the urea extraction beyond 6–9 hr. to about 48 hr. appeared to cause a diminution in the toxicity of the product obtained and this observation may not be unconnected with that of Henderson [1939] that the product obtained by the somewhat lengthy diethyleneglycol extraction of *Bact. typhosum* had been reduced "to a position intermediate between that of the complete antigen of the intact cell and a true hapten". The possibility of a continuous slow enzymic destruction of the complete antigen showing up markedly in these cases of lengthy extraction should be borne in mind since it has been shown by Boivin & Mesrobeanu [1935] that the cells of *Bact. typhi-murium* and *Bact. enteritidis* contain an enzyme exerting precisely such an effect; they showed it to have an optimum *p*H of 7–8, to be inactivated by trichloroacetic acid and to be destroyed by heating to 100° or by trypsin at *p*H 8·5.

Experimental

Bacteria

These were supplied in the form of dried bodies by Professors Topley and Raistrick of the London School of Hygiene and Tropical Medicine. The methods of cultivation and killing were as follows:

Bact. typhi-murium: (A) 24-hr. cultures on nutrient agar; scraped off; acetone-killed. (S) 24-hr. cultures on the synthetic medium described in the preceding paper [Freeman *et al.* 1940]; formalin-killed.

Bact. typhosum (strain R.R.): (A) 24-hr. cultures on nutrient agar in Roux bottles; washed off; acetone-killed.

Bact. typhosum (strain Ty_2): (S) 24-hr. cultures on the synthetic medium; formalin-killed.

Extraction of bacteria with urea solutions

The following description is that of a typical experiment. 12·5 g. of bodies of *Bact. typhosum* (strain Ty_2), grown on the synthetic medium, were ground in a Griffiths tube and stirred for 9 hr. at 38° with 750 ml. of a 2·5 *M* solution of urea in sterile distilled water. The suspension was stored overnight in the ice chest in the presence of toluene and then clarified by passage through a De Laval centrifuge. The liquor retained by the centrifuge bowl (approximately 250 ml.) was displaced with 400 ml. of sterile distilled water, and the slimy residue left on the sides of the bowl was scraped out, treated with alcohol, washed with alcohol and ether and dried in a vacuum desiccator over conc. H_2SO_4; the dry residue amounted to 9·2 g.

The clear pale yellow liquor discharged from the centrifuge was dialysed in collodion sacs under a layer of toluene for 48 hr. against running tap water and for a like period against liberal quantities of distilled water with frequent changes. At the end of the dialysis the solution was paler and slightly opalescent; it was filtered through Chamberland L_2 candles and the filtrate, which retained a trace of opalescence, was evaporated to small bulk (77 g.) below 40° under 18 mm. pressure. During concentration opalescence increased; it increased still further on addition of the theoretical amount of 2 *N* acetic acid required to make the final concentration of acid *N*/10, but no significant sedimentation occurred when the acidified solution was centrifuged on an angle centrifuge at 4000 r.p.m. The supernatant (80 g.) was decanted and filtered alcohol (215 ml.) was added slowly with stirring until the concentration of alcohol reached 68 % by weight. The resultant flocculent white precipitate (F 68) was collected at the centrifuge, washed with alcohol and ether and dried in a vacuum desiccator over conc. H_2SO_4; it amounted to 384 mg. (3·1 % of the weight of dry bacteria used). The supernatant from F 68 was evaporated to small bulk (5–6 ml.) and treated with alcohol (*ca.* 100 ml.); the resultant precipitate (F "95") was collected and dried in the usual way and amounted to 62 mg. Total recovery of material, 9·65 g. (77 % of weight of dry bacteria used).

The results of a number of extraction experiments are summarized in Table 1.

Properties of the various F 68 fractions

The products were all white or pale cream-coloured amorphous solids; they dispersed fairly readily in water to give slightly opalescent solutions which were in many cases sufficiently clear at 1 % concentration to be polarizable through a depth of 5 cm. although accurate estimations of optical rotatory power were not attempted. All the F 68 fractions showed a positive xanthoproteic reaction and (approximately) 1 % solutions gave a strong Molisch reaction, but in no case was a definite protein reaction shown with fresh ninhydrin solution although in the case of the typhoid preparations the reagent gave a faint pink colour on prolonged heating; the biuret reaction was positive though weak in all cases. Esbach's reagent and salicylsulphonic acid (used as 5 % solution in 20 % aqueous Na_2SO_4) gave barely perceptible turbidities of very similar order to those given by an 0·03 % solution of gelatin which was used for comparison side by side with the tests on 1 % antigen solutions; these tests, while they did not indicate the complete absence of intact protein, showed that no significant amounts could be present.

With uranium acetate only the *Bact. typhosum* preparations gave precipitates and these were soluble in citric acid solution [cf. Rouchdi, 1938; Boivin & Mesrobeanu, 1938]. Estimates of the carbohydrate content of the various F 68

Table 1. *The extraction of bacteria with aqueous urea solutions, showing the results of toxicity tests*

Organism	*Bact. typhi-murium*			
	A (i)*	S (i)†	S (ii)	S (iii)
Preparation (F 68):				
Scale of exp. (g. bodies used)	7	12	10	12
Time of extraction at 38° (hr.)	6	48	8·5	9
Molar conc. of urea solution	2·5	2·5	2·5	6·5
% yield of F 68	1·7	5·7	0·5	2·5
Found for F 68:				
R.V. (%) after hydrolysis‡	—	26·6	—	35·3
N (%)‡	—	—	—	4·9
Ash (%)	—	—	—	9·7
Toxicity of F 68:				
Average lethal dose for mice (mg.)	0·23	1·23§	—	0·20

Organism	*Bact. typhosum*						
	Strain R.R.		Strain Ty_2				
	A (ii)	A (iii)	S (iv)	S (v)	S (vi)	S (vii)	S (viii)
Preparation (F 68):							
Scale of exp. (g. bodies used)	20	10	2·5	2·5	12·5	24	30
Time of extraction at 38° (hr.)	45	9	9	9	9	9	9
Molar conc. of urea solution	2·5	2·5	2·5	6·5	2·5	2·5	2·5
% yield of F 68	3	14	3·0	3·5	3·1	3·6	2·9
Found for F 68:							
R.V. (%) after hydrolysis‡	35·6	21·8	—	20·4	23·1	23·1	29·2
N (%)‡	—	9·2	—	—	8·7	7·5	7·0
Ash (%)	—	11·6	—	—	8·3	9·9	12·6
Toxicity of F 68:							
Average lethal dose for mice (mg.)	>8§	<0·46	0·54	—	0·47§ 0·41	—	—

* A signifies "agar-grown" organism.
† S signifies organism grown on "synthetic medium".
‡ These values are *not* calculated on an ash-free basis.
§ A more resistant strain of mice used in these tests.

fractions were obtained by hydrolysing weighed amounts (10–40 mg.) with $2N$ HCl at 100° and then neutralizing carefully with $2N$ NaOH; after dilution to a definite volume, aliquot parts were used in the Hagedorn & Jensen [1923] procedure. The values found are recorded in Table 1 as "R.V. (%) after hydrolysis".

Toxicity of the F 68 fractions

Toxicity tests were carried out by Dr Joyce Wilson at the London School of Hygiene and Tropical Medicine. The fractions were tested by injecting mice intraperitoneally with falling doses from 8 to 0·125 mg. The method used in calculating the average lethal dose was that described by Irwin & Cheeseman [1939]. The results are appended to Table 1.

Immunizing potency of F 68 fractions

Tests of immunizing potency were performed by Dr Joyce Wilson at the London School of Hygiene and Tropical Medicine, using the technique of Raistrick & Topley [1934]; 30 mice were treated and 30 used as controls in each experiment; at the end of the immunization period, all the animals received the same lethal dose of living bacteria, and were observed over a period of 28 days. The results are shown in Table 2.

Table 2. *Showing the deaths out of 30 mice in each group in immunized and control groups*

Immunizing antigen	Infecting organism	Deaths	
		Immunized	Controls
A (i) F 68	*Bact. typhi-murium*	22*	26†
A (ii) F 68	*Bact. typhosum* (strain R.R.)	0	26

* Mean survival time, 16·2 days. † Mean survival time, 11·6 days.

It will be noticed that the *Bact. typhosum* preparation A (ii) F 68 gave full protection to mice although it had lost much of its toxicity, while the highly toxic *Bact. typhi-murium* preparation A (i) F 68 was much less effective. This is quite compatible with the belief that mice should be immunized more readily against *Bact. typhosum* than against *Bact. typhi-murium*, since the latter is a natural mouse pathogen.

SUMMARY

Aqueous solutions of urea extract from bacteria of the *Salmonella* group substances which contain a considerable proportion of carbohydrate and at the most only traces of protein; these substances are highly toxic and confer active immunity on mice.

The author is indebted to Prof. W. W. C. Topley and Prof. H. Raistrick for supplies of bacterial bodies and to Dr Joyce Wilson for carrying out the biological tests. He owes his introduction to their collaboration to Sir Robert Robinson and thanks Messrs Imperial Chemical Industries Ltd. (Dyestuffs Group) for bearing the expenses of the work in this laboratory and for permission to publish the above account of it.

REFERENCES

Anson & Mirsky (1929–30). *J. gen. Physiol.* **13**, 121.
Boivin & Mesrobeanu (1935). *C.R. Soc. Biol., Paris*, **118**, 1671.
—— —— (1938). *C.R. Acad. Sci., Paris*, **206**, 1416.
—— —— & Izard (1938). *C.R. Soc. Biol., Paris*, **127**, 651.
—— —— & Mesrobeanu (1933, 1). *C.R. Soc. Biol., Paris*, **113**, 490.
—— —— —— (1933, 2). *C.R. Soc. Biol., Paris*, **114**, 307.
Burk & Greenberg (1930). *J. biol. Chem.* **87**, 197.
Delafield & Smith (1939). *Brit. J. exp. Path.* **20**, 216.
Freeman, Challinor & Wilson (1940). *Biochem. J.* **34**, 307.
Fuller (1938). *Brit. J. exp. Path.* **19**, 130.
Hagedorn & Jensen (1923). *Biochem. Z.* **135**, 46.
Henderson (1939). *Brit. J. exp. Path.* **20**, 11.
Henderson & Morgan (1938). *Brit. J. exp. Path.* **19**, 82.
Irwin & Cheeseman (1939). *J. Hyg., Camb.*, **39**, 574.
Morgan (1936). *Biochem. J.* **30**, 909.
—— (1937). *Biochem. J.* **31**, 2003.
Neuberger (1938). *Biochem. J.* **32**, 1435.
Raistrick & Topley (1934). *Brit. J. exp. Path.* **15**, 113.
Rouchdi (1938). *C.R. Soc. Biol., Paris*, **128**, 1022, 1024.
Sordelli & Mayer (1931). *C.R. Soc. Biol., Paris*, **107**, 736.
Spiro (1900). *Hoppe-Seyl. Z.* **30**, 182.

46. OBSERVATIONS ON THE DISTRIBUTION OF VITAMIN B_1 IN SOME PLANT FAMILIES

By MAGNUS PYKE

From the Research Laboratories, Vitamins Limited, London

(*Received 15 January 1940*)

The presence of vitamin B_1, at least in small amounts, appears to be almost universal in higher plants; Schopfer [1936], using as his criterion the growth of *Phycomyces*, detected its presence in the leaves of 134 species of plants. Numerous assays obtained by animal feeding tests on vegetable foodstuffs have also been carried out. The widespread distribution of vitamin B_1 seems to imply that it plays a fundamental part in the metabolism of plants. It was therefore thought to be of interest to investigate from a strictly quantitative basis its incidence in certain important plant families.

The occurrence of vitamin B_1 in the outer integuments and germ of cereal grain is well known. Wheat seeds contain some 100 International Units per 100 g. Bran contains 200–300 and germ 600–2000 I.U. per 100 g. Other grains such as barley, rye and oats contain similar amounts. Outside these species, however, the quantitative distribution of vitamin B_1 is not so well established. The development of a modification of the thiochrome technique by which vitamin B_1 could be conveniently and reliably determined has made it possible to investigate its distribution more closely.

The method which has been adopted in this work has been used successfully on a large number of foodstuffs and the good agreement of the results obtained compared with such published figures as are available appears to establish its reliability.

Experimental

A summary of the method used for the determination of vitamin B_1 is given in Table 1. Fuller details have been published elsewhere [Pyke, 1939]. The results obtained represent the total vitamin present and take no account of whether it occurs in the phosphorylated form as cocarboxylase or in the free state.

Table 1. *Summary of method of determining vitamin* B_1

(1) *Digestion with pepsin.* 20 g. sample finely minced or ground; made up to 97·4 ml. with 0·1% pepsin in 0·33% HCl; incubated at 37° overnight

(2) *Digestion with taka-diastase.* 2·6 ml. *N* NaOH added and 100 mg. taka-diastase; incubation continued for a further 5 hr.

(3) *Oxidation of vitamin* B_1 *to thiochrome.* Two 3 ml. centrifuged aliquots added to

(*a*) 2 ml. methanol; 1 ml. 30% NaOH; 1% $K_3Fe(CN)_6$
(*b*) 2 ml. methanol; 1 ml. 30% NaOH

The solutions stirred with N_2; made up to 10 ml. with water; shaken with 13 ml. *iso*butyl alcohol

(4) *Measurement of thiochrome.* 10 ml. portions of *iso*butyl alcohol solutions taken from aliquots (*a*) and (*b*) and 1 ml. ethanol added to give clear solutions. The tubes are held side by side in ultraviolet light and standard *iso*butyl alcohol solution of thiochrome (2 μg. per ml.) added to aliquot (*b*) until the intensity of its fluorescence matches that of aliquot (*a*)

The figures from analysis by this method appear to be reproducible within narrow limits. For example, a single cauliflower was finely minced and 10 samples each of 20 g. were separately analysed. The following results were obtained (I.U. per 100 g.) 45, 45, 45, 46, 46, 48, 48, 48, 48, 48. This gives an average of 47 I.U. per 100 g. and a standard deviation of 1·3 I.U., or 3%. In considering any figures for the vitamin B_1 content of plants, it is important to realize that besides possible discrepancies due to the method of analysis, another factor to be considered is the variation in vitamin B_1 content between different samples of the same plant. Three examples are given in Table 2. Except in the case of apples, the variation appears to be quite slight.

Table 2. *Variation in vitamin B_1 content among different samples of the same materials.* I.U./*100 g.*

Apples		Cauliflower		Potatoes	
Variety unknown	6	Varieties	34	Great Scott, second early	25
James Grieve, dessert	8	unknown	48	Majestic, main crop	27
King Pippin, dessert	15		48	Epicure, first early	29
Miller Seedling, dessert	19		51	Red King, main crop	30
Potts Seedling, cooking	19		53	Variety unknown	30
Quarenden, dessert	19		57		
Warner King, cooking	19				
Worcester Pearmain, dessert	23				

It is interesting now to fit these figures into a wider survey of the vitamin B_1 distribution among the different botanical families within which most of the food plants fall and with which this investigation is primarily concerned. Results are given in Table 3.

Table 3. *Concentrations of vitamin B_1 in plants of different families.* I.U./*100 g.*

Family	Common name and species	Vitamin B_1: Daniel & Munsell [1937]	Vitamin B_1: Present work
	I. Leaves and tops		
Ficoidaceae	New Zealand spinach (*Tetragonia expansa*)	—	21
Compositae	Endive (*Cichorium Endivia*)	—	19
	Globe artichoke (*Cynara Scolymus*)	—	95
	Lettuce (*Lactuca sativa*)	10–22	23
Cruciferae	Cabbage (*Brassica oleracea*)	13–22	23
	Cauliflower (*Brassica oleracea*)	—	50
	Turnip-top (*Brassica campestris* var.)	19	19
	Seakale (*Crambe maritima*)	—	27
Polygonaceae	Sorrel dock (*Rumex acetosa*)	—	25
Umbelliferae	Celery (*Apium graveolens*)	—	15
	Angelica (*Archangelica officinalis*)	—	38
	Rock samphire (*Crithmum maritimum*)	—	48
	Alexanders (*Smyrnium Olusatrum*)	—	38
	II. Roots, bulbs, rhizomes and tubers		
Chenopodiaceae	Beetroot (*Beta vulgaris*)	—	6
Compositae	Chicory (*Cichorium Intybus*)	—	25
	Jerusalem artichoke (*Helianthus tuberosus*)	—	76
Convolvulaceae	Sweet potato (*Ipomoea Batatas*)	22–23	34
Cruciferae	Radish (*Raphanus sativus*)	7–9	13
	Swede (*Brassica campestris* var.)	—	25
	Turnip (*Brassica campestris* var.)	<12	11
Leguminosae	Tuberous bitter-vetch (*Lathyrus montanus*)	—	57

Table III (*cont.*)

Family	Common name and species	Vitamin B_1: Daniel & Munsell [1937]	Vitamin B_1: Present work
	II. Roots, bulbs, rhizomes and tubers (*cont.*)		
Liliaceae	Leek (*Allium Ampeloprasum*)	18	51
	Onion (*Allium Cepa*)	5	8
	Ramsons (*Allium ursinum*)	—	Nil
	Solomon's seal (*Polygonatum multiflorum*)	—	76
	Spiked Ornithogalum (*Ornithogalum pyrenaicum*)	—	57
Solanaceae	Potato (*Solanum tuberosum*)	8–40	30
Umbelliferae	Carrot (*Daucus Carota*)	6–33	15
	Parsnip (*Peucedanum sativum*)	26	38
	Angelica (*Archangelica officinalis*)	—	48
	Alexanders (*Smyrnium Olusatrum*)	—	21
	III. Seeds and nuts		
Coniferae	Pine kernels (*Pinus* spp.)	—	162
Cucurbitaceae	Pumpkin, whole seed (*Cucurbita maxima*)	—	76
Betulaceae	Barcelona nuts (*Corylus maxima*)	—	38
	Cob nuts (*Corylus hybrida*)	—	76
Lecythidaceae	Brazil nuts (*Bertholletia excelsa*)	—	340
Leguminosae	Butter beans (*Phaseolus lunatus*)	—	140
	Lentils (*Lens esculenta*)	—	170
	Peanuts (*Arachis hypogaea*)	73–220	228
	Green peas (*Pisum sativum*)	17–100	120
Oleaceae	Ash, whole fruit (*Fraxinus excelsior*)	—	114
	IV. Fruit		
Ananaceae	Pineapple (*Ananas comosus*)	25	10
Cucurbitaceae	Cucumber (*Cucumis sativus*)	—	15
	Marrow (*Cucurbita Pepo*)	—	Nil
	Water-melon (*Citrullus vulgaris*)	—	23
Rosaceae	Apple (*Malus pumila*)	3–9	6–23
	Pear (*Pyrus communis*)	2–14	18
	Apricot (*Prunus Armeniaca*)	9	20
	Damson (*Prunus domestica* var.)	—	38
	Greengage (*Prunus domestica* var.)	—	66
	Peach (*Prunus persica*)	3	6
	Plum (*Prunus domestica* var.)	—	66
	Blackberry (*Rubus* sp.)	—	13
	Haws: (*Crataegus*) green (1 variety)	—	42
	(*Crataegus*) red (1 variety)	—	19
	Rose hips: red 5 varieties	—	Nil
Rutaceae	Lemon (*Citrus Limon*)	—	6
	Orange (*Citrus sinensis*)	33	34
Solanaceae	Tomato (*Lycopersicum esculentum*)	7–13	15
Vitaceae	Currant	—	10
	Grape	4–11	11
	Raisin	23–35	29
	Sultana	—	29
	(*Vitis vinifera* vars.)		

Discussion

The uniformity of the vitamin B_1 content of leaves and tops is striking. This is perhaps not unexpected if it is assumed, as may well be the case, that vitamin B_1 is synthesized in the leaves and that this synthesis is a side branch of the general metabolism of the plant hinging about the activity of chlorophyll. Be

that as it may, a far greater general uniformity of vitamin B_1 concentration exists in the leaves than elsewhere in the plant.

Turning to the vitamin B_1 content of the parts below ground, while there is little general agreement between different families, a similarity in concentration exists to some extent within each family. In the Liliaceae the differences which appear may be due in part at least to the comparison of different organs. However, in the genus *Allium* the colourless bulbs of *A. Cepa* and *A. ursinum* contain little or no vitamin while the leek contains an appreciable amount, perhaps because it consists of partly expanded leaves with a considerable amount of chlorophyll. The accumulation of the vitamin in a particular organ may be a character of specific or possibly generic value since the colourless bulbs of *Ornithogalum pyrenaicum* contain almost as much as the rhizomes of *Polygonatum multiflorum*. Although, compared with the high values in the Leguminosae, the potato appears to contain little vitamin B_1, nevertheless from the point of view of human nutrition it provides very substantial amounts due to the quantity which can be eaten conveniently.

Although it has not been possible to examine a sufficient number of examples within each family to be able to draw many definite conclusions it seems probable that in most cases the seeds contain the highest proportion of vitamin B_1 in the plant. Also, with the exception of the outstandingly high value of Brazil nuts, representing the Lecythidaceae, legumes appear in general to contain the highest amounts of the vitamin. Indeed the concentration in the seeds of this family is surprisingly uniform. The higher value in peanut and the lower in green pea might logically be attributed to a mere difference in moisture content.

In the case of fruit, the important family of Rosaceae presents an opportunity for speculation. Whereas a green example of *Crataegus* contained 42 I.U. per 100 g., a red sample only showed 19. Rose hips in the red condition, moreover, possessed no vitamin B_1 whatever. It seems possible that this decrease in vitamin B_1 content is due to increasing degrees of ripeness as the climacteric is approached. If this hypothesis is correct it might offer an explanation of the variation in activity of the different varieties of apples examined.

In addition to the figures obtained during the course of this investigation a second column has been included in the table of results made up of figures collected by Daniel & Munsell [1937]. These authorities have collected a very large number of published results. In their paper the figures are given in Sherman units. Conversion factors of either $\frac{1}{2}$ or $\frac{1}{4}$ are suggested for transforming Sherman units into International Units. In the present table Daniel and Munsell's figures have been converted into I.U. by dividing them by three. It will be seen that the thiochrome method used in the present work gives results which compare quite well with these published figures obtained from biological assay.

Summary

1. The vitamin B_1 content of different parts of a number of plants from certain important botanical families has been determined by the thiochrome technique. The results obtained compare favourably with published figures where these are available.

2. In general the vitamin B_1 content is highest in seeds. In most leaves the concentration of vitamin B_1 amounts to a constant value of about 25 I.U. per 100 g., regardless of the botanical family.

3. The possibility is tentatively suggested that variation in vitamin B_1 content of fruits of the Rosaceae is due to the degree of ripeness.

LINDA HALL LIBRARY
KANSAS CITY, MO.

The author wishes to take this opportunity of thanking the Director of the Royal Botanic Gardens, Kew, for providing specimens of plants. He also wishes to acknowledge his indebtedness to Dr R. Melville for his advice and help with the botanical classification.

REFERENCES

Daniel & Munsell (1937). *Misc. Publ. U.S. Dep. Agric.* no. 275.
Pyke (1939). *J. Soc. chem. Ind.* **58**, 338.
Schopfer (1936). *Arch. Mikrobiol.* **7**, 156.

47. THE SPECTROSCOPIC DETECTION OF VITAMIN E IN THE TISSUES OF THE RAT[1]

By THOMAS MOORE and KRISHNA RAO RAJAGOPAL[2]

From the Nutritional Laboratory, University of Cambridge, and Medical Research Council

(*Received 29 January 1940*)

Spectroscopic studies of concentrates of vitamin E prepared from wheat germ and cotton seed oils led to the detection of an absorption band at about 294 mμ [Bowden & Moore, 1933; Olcott, 1934; Martin *et al.* 1934; Drummond *et al.* 1935]. This band was later found to be characteristic of pure tocopherol whether natural [Evans *et al.* 1936] or synthetic [Karrer & Demole, 1938].

The detection and estimation of vitamin E in tissues by the spectroscopic method is, however, complicated by several difficulties. The extraction of the vitamin from the tissues may involve destruction or loss, particularly if saponification is included in the procedure [Emmerie & Engel, 1939]. On the other hand, if saponification is omitted substances other than vitamin E which contribute substantial absorption at 294 mμ are included. Moreover, when tocopherol is acetylated its absorption is decreased in intensity, with alteration of the maximum to about 285 mμ. The presence of tocopherol in the tissues in esterified form, a possibility which cannot be ruled out, might therefore make its spectroscopic detection more difficult. Finally the absorptions of α-tocopherol ($E^{1\%}_{1\,\mathrm{cm.}}$ 294 mμ = *ca.* 77) and α-tocopherol acetate ($E^{1\%}_{1\,\mathrm{cm.}}$ 285 mμ = *ca.* 42) are much less intense than that of vitamin A ($E^{1\%}_{1\,\mathrm{cm.}}$ 328 mμ = *ca.* 1800). The strong absorption of vitamin A would make the spectroscopic detection of vitamin E impossible in the liver without the careful removal of the vitamin A by processes which might cause loss of vitamin E.

The rats used in the present work received diets containing vitamin A in amounts so adjusted as to permit maintenance, while being inadequate to allow storage in the liver. Spectroscopic examinations were made on tissues from animals deficient in vitamin E, and for comparison from others which had been given large amounts of wheat germ or synthetic tocopherol. The results obtained indicate that vitamin E may be detected spectroscopically in the fat of specially prepared animals. At its present stage of development, however, the method cannot be recommended for routine estimations.

Experimental

Material. Tissues were examined from rats under the following conditions: (1) After restriction to a diet deficient in vitamin E (light white caseinogen 25%, rice starch 50%, lard 20%, salt mixture 5%+dried yeast 15%, radiostol (vitamin D) one drop per week). The casein gave enough vitamin A for maintenance or slow growth, but not enough to allow storage in the liver. (2) After

[1] A brief report of some of the results described in this paper was included in a communication by Moore *et al.* [1939] at the recent Symposium on Vitamin E held by the Food Group of the Society of Chemical Industry.

[2] Rockefeller Research Fellow.

restriction to the diet deficient in vitamin E followed by dosing with synthetic *dl*-α-tocopherol. (3) After maintenance for long periods on a diet containing wheat germ (casein, alcohol-extracted 20 %, wheat germ 60 %, arachis oil 15 %, salts 5 %+dried yeast 10 %). Even this large amount of wheat germ did not cause measurable storage of vitamin A in the liver.

Methods. The rats were killed by coal gas. The tissues were dissected and digested by mincing and heating with 5 % aqueous KOH. Digestion was usually complete in about 2 hr. Alcohol was then added to the digest and the fat extracted with ether. The extract was washed, evaporated, saponified for about 1 min. with hot alcoholic KOH and the unsaponifiable fraction separated in the usual way. Two precautions were necessary to prevent undue spectroscopic absorption in blank experiments. Ether of sp. gr. 0·730 (B.D.H.) which is satisfactory for use in vitamin A determinations by the $SbCl_3$ method [Davies, 1933] had to be redistilled. The use of anhydrous Na_2SO_4 in drying the ethereal extracts caused considerable absorption and was abandoned. In some experiments the absorption was measured before saponification of the fat. In a few instances duplicate determinations were done on extracts prepared by grinding the tissues with anhydrous Na_2SO_4 and extracting by the Soxhlet method, the extracts being carefully washed to remove Na_2SO_4 before evaporation.

Solutions of the extracts in alcohol or *cyclo*hexane were examined with a Hilger H 290 spectrophotometer. The colorimetric method of Emmerie & Engel [1938], using $FeCl_3$ and α-α-dipyridyl, was also applied during the later stages of the investigation.

Calculation of results. Absorption data have been calculated on the weight of fresh tissue, without reference to the fat content, and are expressed as $E^{100\ \%}_{1\ \mathrm{cm.}}$ instead of the usual $E^{1\ \%}_{1\ \mathrm{cm.}}$ in order to adjust the decimal point to a convenient position. Results are given to two figures, although the second is of doubtful significance if the first is high.

Comprehensive dissections. In early experiments the dissections included brain, heart, lung, kidneys, liver, spleen, intestines, muscle, bone, blood, thymus, suprarenals, pituitary, testes, seminal vesicles, uterus, ovaries, fat deposits, urine and faeces. Such experiments were done on young rats reared for 2 months on the diet deficient in vitamin E, on a similar rat killed 48 hr. after receiving 60 mg. of synthetic *dl*-α-tocopherol, and on an adult rat which had received the diet containing 60 % of wheat germ for 2 years. Extractions were by the standard method including saponification. Even with the rat given wheat germ, which showed the absorption band of vitamin E in its fat deposits, the absorption of most of the small organs was too low to be measured. The absorption of large organs, with the possible exception of muscle, gave no indication of the presence of vitamin E. In all these rats, however, absorption due to unidentified substances was shown by some tissues, usually as an inflexion at about 280 mμ. The intestinal tract, cleared of its contents, showed an absorption band at 275–280 mμ. Faeces sometimes showed much general absorption, but the intensity varied greatly even with rats receiving the same diet. With faeces much higher results were obtained by extracting the fat by the Soxhlet method than by our usual procedure of digestion with alkali.

In later experiments, attention was usually confined to the fat deposits and liver. This organ was selected as being the main site of storage for some other vitamins and as yielding the next most highly absorbing extracts after fat and faeces.

Storage fats. Spectroscopic and colorimetric data on storage fat are given in Table 1. Absorption bands at 294 mμ were seen in 3 out of 4 fats taken from

Table 1. *Spectroscopic and colorimetric data on the unsaponifiable fractions of the fat deposits of rats given diets with and without vitamin E*

No. of rat	Treatment	Site of deposits I.P. = intraperitoneal S.C. = subcutaneous	$E^{1\%}_{1\,cm.}$ 294 mμ	$E^{1\%}_{1\,cm.}$ 270 mμ	Nature of spectroscopic absorption	Max. tocopherol mg. per 10 g. by spectroscopic method	Tocopherol mg. per 10 g. by Emmerie's method
		Diet deficient in vitamin E					
1	Deficient diet for 2 months	I.P.	0·88	1·28	Inflexion 275 mμ (Fig. 1)	—	—
2	Deficient diet for 4 months	I.P.	0·35	0·44	Inflexion 290–270 mμ	0·45	0
		Diet deficient in vitamin E with doses of tocopherol					
3*	Tocopherol acetate, 3 mg. per week for 4 months	I.P.	0·37	0·43	Slight inflexions 290 and 330 mμ	—	—
4	Tocopherol acetate, 25 mg. over 5 days	I.P.	0·93	1·4	? Slight inflexion 294 mμ	1·2	0·06
		S.C.	1·1	1·9	No inflexion	1·4	0·3
5	Killed 48 hr. after single dose 64 mg. tocopherol	I.P.	0·91	1·8	No inflexion	—	—
		S.C.	1·2	2·0	No inflexion	—	—
		Diet with 60% of wheat germ					
6†	Wheat germ diet for 2 months	I.P.	0·27	0·35	Slight inflexion 280 mμ	—	—
		S.C.	2·0	2·6	Slight inflexion 280 mμ	2·6	4·1
7–11 mixed	Wheat germ diet for 3 months	I.P.	1·1	0·81	Shallow band 294 mμ	1·4	0·81
12‡	Wheat germ diet for 2 years	I.P.	5·6	3·2	Sharp band 294 mμ (Fig. 1)	—	—
13	Wheat germ diet for 2 years	I.P.	3·0	3·2	Inflexion 294 mμ	—	—
		S.C.	2·5	1·7	Band at 294 mμ	—	—

As a guide to the nature of the absorption curves data for $E^{1\%}_{1\,cm.}$ at 294 and 270 mμ are given. The sharpness of absorption due to vitamin E is indicated by the degree of excess of *E* 294 over *E* 270 mμ.

* Rat No. 3 differed from all others in receiving a basal diet containing the same components in slightly different proportions and supplemented by 1 drop of halibut liver oil per week.

† Preliminary extraction of rats by Soxhlet extraction with ether. Colour test done on untreated subcutaneous fat, solution rather turbid.

‡ Muscle from rat No. 12 showed a marked inflexion at 294 mμ. This may have been due to adhering fat.

rats which had been given wheat germ. The sharpest and most intense band was found in the intraperitoneal fat of a rat which had been given wheat germ for about 2 years (Fig. 1). In fat from rats which had received the diet deficient in vitamin E, even when supplemented by tocopherol, the absorption in the region of 294 mμ was marked by no more than a slight inflexion. It may be noted that rat No. 3 was given tocopherol for a prolonged period, but at a level much lower than in the diet with wheat germ. Rats Nos. 4 and 5 were given large doses of tocopherol, but only for short periods.

The colorimetric method of Emmerie & Engel was described at a late stage in this investigation, and unfortunately was not available at the time of killing the rats (Nos. 12 and 13) which showed the highest reserves of vitamin E by the spectroscopic method. With the mixed intraperitoneal fat of rats Nos. 7–11 the colour reaction was positive. These rats had received wheat germ for 3 months. The reaction was also positive with the subcutaneous fat of rat No. 6, which had been given wheat germ for 2 months. No reaction was obtained with the

intraperitoneal fat of a rat (No. 2) which had received a diet deficient in vitamin E. The reaction was also insignificant with the intraperitoneal fat of a similar rat (No. 4) which had received large doses of tocopherol over a short period, although the subcutaneous fat of this animal gave a slight reaction.

In general the numerical agreement between the maximum values given by the spectroscopic method and results given by the colorimetric method was poor, indicating the presence of a considerable amount of non-specific absorption in most instances. In the one case (rats Nos. 7–11) in which both a definite absorption band and a positive colour reaction were observed, however, the results by the two methods were reasonably consistent, on the assumption that vitamin E contributed slightly more than half of the spectroscopic absorption. This would be in agreement with the shallow shape of the absorption band. With rat No. 6 the fact that the tocopherol content of the subcutaneous fat found by the colorimetric method exceeded the maximum value that could be obtained by the spectroscopic method may have been due to a slight cloudiness of the solution in the colour test.

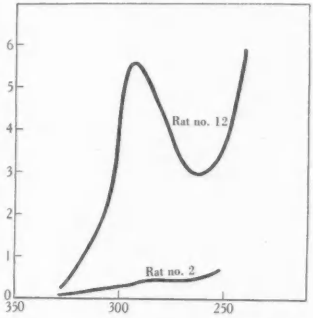


Fig. 1. Spectroscopic absorption of the unsaponifiable fraction of the intraperitoneal fat of a rat (Table 1, No. 12) given wheat germ for 2 years (upper curve). The maximum is at 294 mμ, the position characteristic of vitamin E. The lower curve is for the corresponding fraction from a rat (Table 1, No. 2) which had been restricted to a diet deficient in vitamin E.

In some instances the fats were examined spectroscopically before saponification. The absorption was always much greater than that of the unsaponifiable fractions, being at least 3 times greater in the region of 294 mμ. This position is at the foot of the steep incline in absorption with decreasing wave-length which is seen in most fats. A distinct inflexion at about 275 mμ was present in some instances. By the colour reaction the mixed untreated fat of rats Nos. 7–11 gave a value of 0·88 mg. per 10 g. of tissues, which is only slightly higher than the 0·81 mg. found for the unsaponifiable matter.

Liver. Spectroscopic and colorimetric data for the unsaponifiable matter of liver fat are given in Table 2. In no instance was there any evidence of selective absorption at 294 mμ. The general absorption of the extracts was possibly slightly greater for rats given wheat germ or tocopherol than for rats receiving the diet deficient in vitamin E.

Colour reactions were given by all livers examined. The differences between results for different rats were so small as to be of doubtful significance, and certainly did not reflect the widely varied tocopherol intakes. With the rats receiving wheat germ the tocopherol content given by the colour reaction was about half the maximum value found by the spectroscopic method. The absence of selective absorption at 294 mμ indicates that tocopherol, if present, must contribute only a fraction of the absorption at this position. There is, therefore, no inherent inconsistency between the spectroscopic and colorimetric data for these animals. For the same reason the data by the two methods for the rats on the diet deficient in vitamin E, with and without tocopherol, which are in good numerical agreement, are difficult to reconcile with the specificity of both the methods. Unidentified substances may contribute to the colour reaction.

Table 2. *Spectroscopic and colorimetric data on the unsaponifiable fractions of the liver fats of rats given diets with and without vitamin E*

No. of rat	Treatment	$E^{100\,\%}_{1\,cm.}$ 294 mμ	$E^{100\,\%}_{1\,cm.}$ 270 mμ	Nature of spectroscopic absorption	Max. tocopherol mg. per 10 g. by spectroscopic method	Tocopherol mg. per 10 g. by Emmerie's method
	Diet deficient in vitamin E					
14	Deficient diet for 2 months	0·49	0·92	Inflexion 280 mμ	—	—
2	Deficient diet for 4 months	0·6	1·5	No inflexion	0·78	0·64
	Diet deficient in vitamin E with doses of tocopherol					
4	Tocopherol acetate 25 mg. over 5 days	0·74	0·98	Slight inflexion 275 mμ	0·97	0·95
5	Killed 48 hr. after single dose 64 mg. tocopherol	1·1	1·5	Slight inflexion 285 mμ	—	—
	Diet with 60% of wheat germ					
6	Wheat germ diet for 2 months	0·98	1·3	No inflexion	1·3	0·73
7–11 mixed	Wheat germ diet for 3 months	1·1	1·6	No inflexion	1·4	0·69
12	Wheat germ diet for 2 years	1·4	1·8	Inflexion 285 mμ	—	—

Table 3. *Spectroscopic and colorimetric data on untreated liver fats of rats given diets with and without vitamin E*

No. of rat	Treatment	$E^{100\,\%}_{1\,cm.}$ 294 mμ	280 mμ	250 mμ	Nature of spectroscopic absorption	Intensity of purple colour on saponification	Max. tocopherol mg. per 10 g. by spectroscopic method	Tocopherol mg. per 10 g. by Emmerie's method
	Diet deficient in vitamin E							
14	Deficient diet for 2 months	—	—	—	—	Very faint	—	—
2	Deficient diet for 4 months	3·1	6·9	5·4	Band 275 mμ	Strong	16	0·59
	Diet deficient in vitamin E with doses of tocopherol							
4*	Tocopherol acetate 25 mg. over 5 days	2·3	4·9	6·1	Shallow band 275 mμ. High absorption in far U.V.	Faint	12	1·2
5	Killed 48 hr. after single dose 64 mg. tocopherol	—	—	—	—	Very faint	—	—
	Diet with 60% of wheat germ							
6	Wheat germ diet for 2 months	1·8	5·8	3·2	Band 275 mμ	Strong	14	0·68
7–11 mixed	Wheat germ diet for 3 months	2·0	6·5	3·0	Band 275 mμ	Intense	15	1·1
13	Wheat germ diet for 2 years	—	—	—	—	Intense	—	—

* Fat obtained by Soxhlet extraction.

When the liver fats were examined without saponification (Table 3) a band at about 275 mμ was always observed. This position is between the maximum at 285 mμ for tocopherol acetate and the main maximum at 271 mμ for the highly absorbing substances obtained by Evans *et al.* [1936] from tocopherol by treatment with $AgNO_3$. The band at 275 mμ varied in sharpness in different extracts, but remained nearly constant in intensity, being slightly more intense for the rat which had received the deficient diet than for the others which had been given wheat germ or tocopherol. The assumption that this band is actually due to an ester of tocopherol seems untenable, since it would imply the presence of about 12 mg. of tocopherol in the liver of the animal which had received the diet deficient in vitamin E. The band at 275 mμ did not appear in the unsaponifiable fraction after saponification.

It may be mentioned that the saponification of the liver fats was always accompanied by the development of a transient bright purple colour of varying intensity. This colour reaction suggests the presence of vitamin K. No evidence of the complex absorption spectrum with maxima at 248, 261, 270 and 328 mμ as found by Dam *et al.* [1939] for this vitamin could be distinguished in the fat before saponification. Preliminary tests with chicks in collaboration with Miss E. M. Cruickshank, however, suggested that the liver fat from rats given wheat germ possessed some activity in promoting the clotting of blood. The possibility of the association of the disappearance of the 275 mμ absorption band with the development of the purple coloration may be kept in mind. The comparatively constant intensity of the absorption band must, however, be contrasted with the great variations in intensity of the purple coloration.

With rat No. 6 grinding of the liver tissues with anhydrous Na_2SO_4 followed by Soxhlet extraction gave an extract which absorbed nearly twice as strongly as the extract obtained by digestion with alkali. The sharpness of the absorption band at 275 mμ was much reduced, suggesting that the increased absorption was due to the inclusion of additional substances with non-selective absorption rather than to increased stability of the substance absorbing at 275 mμ.

Results by the colorimetric method on the unsaponified liver fats were usually in fair agreement with those obtained after saponification.

Discussion

Interpretation of the above results is complicated by the difficulties which have already been mentioned. The anomalous behaviour of tocopherol towards saponification under different conditions undoubtedly presents the most serious problem. Saponification, both cold and hot, was employed at various stages in the original concentration of vitamin E by Evans & Burr [1927]. In the present work sharp absorption bands at the position characteristic of vitamin E were shown by concentrates from storage fat which had been submitted to saponification with hot alcoholic KOH. On the other hand the work of Emmerie & Engel [1939] has shown that pure tocopherol is much less stable to alkali than previously supposed. We have ourselves found that both with synthetic *dl*-α-tocopherol and a concentrate prepared from wheat germ a brief hot saponification may cause 50 % deterioration as measured spectroscopically. Probably the stability of tocopherol towards KOH is influenced by such factors as the excess of KOH used, access to O_2 and the presence of other substances.

It would, therefore, seem permissible to accept the positive results obtained as qualitatively correct, while regarding negative results with caution. The band at 294 mμ shown by the unsaponifiable matter of the storage fat of rats given wheat germ indicates that significant amounts of vitamin E may be

accumulated. The quantity of about 5 mg. found in rat No. 12 is large when compared with the dose (about 2 mg.) necessary for fertility in the female, but represents only about the amount ingested daily in the wheat germ. Since the latter was given for about 2 years only about $\frac{1}{700}$ part of the tocopherol ingested was stored. It may also be noted that the spectroscopic absorption of the fat was much greater in rats which had been given wheat germ for 2 years than in those which had received the wheat germ for 2–3 months. The storage of vitamin E, at least as measured by the 294 mμ absorption band, therefore seems inefficient and slow.

With regard to the other tissues of the rats given wheat germ, and to all tissues of rats given the diet deficient in vitamin E with and without supplements of tocopherol, it must be assumed either that vitamin E was present in amounts too small to survive the processes of extraction, or that for some reason it was present in less stable form. The first explanation is in accordance with the early biological tests of Evans & Burr [1927], who failed to find any animal tissue that was a particularly rich source of vitamin E.

Our inability to trace large doses of tocopherol after ingestion by the rat is in agreement with the experience of Drummond [1939] who suggests that tocopherol may be changed in the tissues, probably with opening of the oxygen ring. Evans *et al.* [1936] have also shown that tocopherol may undergo changes in its chemical structure and absorption spectrum *in vitro* without complete loss of biological activity. The possibility that tocopherol, except in the storage fat, is distributed in the tissues in changed form cannot, therefore, be excluded. We have, however, been unable to associate the intensity of any of the unfamiliar absorption bands encountered in our experiments with the level of the tocopherol intake. The contrast between the storage of vitamin E by rats receiving the diet containing wheat germ and the failure to store by rats given tocopherol might suggest that the vitamin is present in wheat germ in more available form, but there is probably no need to seek an explanation beyond the quantitative superiority of the amounts of vitamin E given as wheat germ.

The purple colour given by liver fat on saponification is of interest in the present work in view of the points of similarity between members of the E and K vitamin groups, which differ chiefly in being derived from duroquinone and naphthoquinone nuclei respectively. If the purple chromogen in liver is in fact a vitamin K, its concentration in liver and absence from storage fat presents a striking contrast with the distribution of the chemically similar vitamin E.

In our limited experience with the colorimetric method of Emmerie & Engel the results for storage fats were qualitatively consistent with the spectroscopic data. With liver extracts our results suggest that the colour reaction may be given by substances other than tocopherol. It was stated by the originators of the method that reactions are also given by carotene and vitamin A, but these were not present in amounts sufficient for detection by yellow colour or the $SbCl_3$ method.

Summary

1. Rats were kept on diets low in vitamin A to prevent the accumulation of reserves of vitamin A, and their tissues were then examined for vitamin E by the spectroscopic method.

2. The investigation was complicated by the possible effects of the processes of extraction, and particularly the use of alkali for digestion and saponification, on the stability of the vitamin. Treatment designed to conserve the vitamin led to the inclusion in the extract of other absorbing substances.

3. Even when alkali was used for digestion and saponification the absorption band at 294 mμ which is characteristic of vitamin E was shown by the depot fat of rats which had been given much wheat germ over long periods. The vitamin was not detected spectroscopically in other tissues.

4. When large doses of *dl*-α-tocopherol, or its acetate, were given to rats over short periods no clear spectroscopic evidence of its storage in the tissues was obtained.

5. In some tissues, even from rats not receiving vitamin E, unidentified substances were present which had absorption near the maximum for esterified tocopherol. Thus, liver fat showed before saponification a relatively intense band at 275 mμ. The intestines also showed a band at about 280 mμ. This band also seemed to be uninfluenced by the dietary history, but differed from the band in liver in resisting saponification.

6. In a few experiments the spectroscopic data were compared with results by the colorimetric method of Emmerie & Engel. In the fat deposits, in qualitative agreement with the spectroscopic results, the vitamin was only detected in significant amounts in rats which had received wheat germ. In liver the amounts of vitamin E present according to the colour test showed little variation with dietary history. Comparison with the spectroscopic data suggested that in liver substances other than tocopherol may have contributed to the colour reaction.

7. On saponification rat-liver fats gave a transient bright purple colour reminiscent of vitamin K.

Our thanks are due to Dr L. J. Harris for his valuable criticism and to Messrs Roche Products, Ltd., for generous gifts of tocopherol.

REFERENCES

Bowden & Moore (1933). *Nature, Lond.*, **131**, 512; **132**, 204.
Dam, Geiger, Glavind, Karrer, Rothschild & Salmon (1939). *Helv. chim. Acta*, **22**, 310.
Davies (1933). *Biochem. J.* **27**, 1770.
Drummond (1939). Vitamin E, A Symposium held under the auspices of the Food Group (Nutrition Panel) of the Society of Chemical Industry, London. Printed by Heffer, Cambridge, p. 27.
—— Singer & MacWalter (1935). *Biochem. J.* **29**, 456.
Emmerie & Engel (1938). *Nature, Lond.*, **142**, 873.
—— —— (1939). *Soc. chim. néerl.* **58**, 895.
Evans & Burr (1927). *Mem. Univ. Calif.* no. 8.
—— Emerson & Emerson (1936). *J. biol. Chem.* **113**, 319.
Karrer & Demole (1938). *Schweiz. med. Wschr.* **68**, 954.
Martin, Moore, Schmidt & Bowden (1934). *Nature, Lond.*, **134**, 214.
Moore, Martin & Rajagopal (1939). Vitamin E, A Symposium held under the auspices of the Food Group (Nutrition Panel) of the Society of Chemical Industry, London. Printed by Heffer, Cambridge, p. 41.
Olcott (1934). *J. biol. Chem.* **105**, proc. lxv.

48. ASSESSMENT OF THE LEVEL OF NUTRITION. URINARY EXCRETION OF ANEURIN AT VARYING LEVELS OF INTAKE[1]

BY Y. L. WANG AND JOHN YUDKIN

From the Nutritional Laboratory, University of Cambridge and Medical Research Council

(*Received 1 February 1940*)

THE estimation of aneurin in the urine was suggested by Harris & Leong [1936] as a method of assessing the body "reserves" of the vitamin. Using the bradycardia method of Birch & Harris [1934], the urinary output was found to be dependent on the past intake, being low, for example, in beriberi and in conditioned deficiencies [Harris *et al.* 1938]. The procedure involved the determination of the "resting level" of excretion and the response to test doses. Since then, many investigators, using either the bradycardia method, or the thiochrome method of Jansen [1936], have measured the urinary excretion of aneurin and confirmed the fact that it is correlated with the state of nutrition of the body. Westenbrink & Goudsmit [1938] found low excretions in women during pregnancy. Schroeder [1939] investigated many patients suffering from a variety of diseases and in the majority found low excretions of aneurin. Wang & Harris [1939] also found low excretions in conditioned deficiencies attributed to anorexia or faulty absorption of the vitamin.

According to most observers, a diet adequate in aneurin is associated with a daily output of 100 μg. or more of the vitamin. Wang & Harris [1939] found the daily excretion of most of the normal individuals whom they examined to lie between 150 and 240 μg. with a maximum of 480 μg. and a minimum of 90 μg. The values obtained by Ritsert [1938] lay between 110 and 510 μg., whilst Schroeder [1939] obtained values of 100–400 μg. Westenbrink & Goudsmit [1938] are of the opinion that individuals excreting over 100 μg. of aneurin are saturated with the vitamin. Wang & Harris [1939] suggested, as provisional standards, that excretions below about 90 μg., combined with delayed response to test doses, could be regarded as evidence of a "sub-average", and probably a "sub-normal", level of nutrition.

For the purpose of assessing the level of nutrition in regard to aneurin, the object to be aimed at is to determine the presumed intake of the vitamin from measurements of the quantity excreted. For this to be possible, it is necessary, as pointed out by Wang & Harris [1939], to have "standard reference curves" relating the amount of aneurin excreted to the intake of the vitamin under standardized conditions, and also to know whether variations between individuals are small enough to allow of the use of such reference values.

The work so far reported on the relation between intake and output has been mainly in the nature of determining the excretions in persons on normal but unspecified diets with and without various supplements of aneurin. For example, the excretion has been determined after oral administration of 5 mg.

[1] Communicated to the Biochemical Society 9 December 1939 [Wang & Yudkin, 1939].

or more of the vitamin by Westenbrink & Goudsmit [1938] and by Sciclounoff [1939], whilst Schroeder [1939] estimated the urinary output after similar large doses injected intravenously. The results of Jolliffe *et al.* [1939], who measured the excretion on a well-controlled deficient diet before and after oral ingestion of aneurin, are discussed in more detail below.

The work to be described was undertaken in order to determine whether it was possible to obtain these "standard reference values" for the excretion of aneurin. We have measured the amounts excreted over a wide range of intake, ranging from definite deficiency (about 100 μg. daily) to distinctly high levels (over 2 mg. daily). A special study was made of the level of excretion with an intake conforming to the reputed daily requirements (300 I.U. or 900 μg.). Lastly, the effects were studied of exercise and of variations in the proportions of fat and carbohydrate in the diet.

Experimental

The subjects of the experiments were four healthy men. Three of these—G. G. B., age 23, Y. L. W., age 31 and J. Y., age 29—were research workers and the fourth—S. A. C., age 24—a laboratory technician. Their weights were 58, 65, 67 and 72 kg. respectively. The intake of aneurin was controlled by taking a basal deficient diet and adding at intervals known supplements of pure aneurin hydrochloride ("Benerva" in ampoules).

The basal diet consisted of well-washed, autoclaved polished rice, corned beef, herrings (fresh cooked, or in its various cured forms), small amounts of white bread, butter, milk steamed for 2 hr. at *p*H 9, strawberries, raspberries and chocolate. Supplements of ascorbic acid and of capsules containing vitamins A and D were also taken. The caloric value of the diet was sufficient to meet the bodily requirements of each subject, since their weights did not alter appreciably throughout the period of the experiment. Although the diet was not weighed each day, the amounts of the various items taken daily by each individual were fairly constant and did not differ greatly for the four subjects. The approximate quantities of the items in the daily diet were as follows: rice 150 g., herrings 300 g., white bread 100 g., milk 400 ml., butter 20 g., sugar 50 g., chocolate 100 g., strawberries or raspberries 150 g.

The amount of aneurin in this diet is 100–150 μg., calculated from the tables of Fixsen & Roscoe [1937–8] and also in most cases confirmed by us, using a modification of the method of Wang & Harris [1939]. No attempt has been made to allow for the vitamin-sparing action of the fat in this diet. It appears from experiments to be described below that the excretion of aneurin is little, if at all, affected by the fat content of the diet.

The daily output of aneurin was measured by the method of Wang & Harris [1939]. The estimations were carried out by each of us independently and almost invariably agreement was obtained within 10% for the higher values and 20% for the very low values.

(1) *Variation of excretion with intake*

Three subjects—S. A. C., Y. L. W. and J. Y.—took part in this experiment. Before the deficient diet was begun the "resting" excretion of each subject on his ordinary diet was determined for several days. The deficient diet without addition of aneurin was taken for 10–14 days and then supplements of 600, 1200 and (in 2 cases) 1800 μg. were in turn taken daily for a period of 4–6 days each. The supplement was taken immediately before or after the main (midday) meal.

The effect of a still higher level of intake was studied on a normal diet with a supplement of 2 mg. daily. The daily excretions of aneurin in this experiment are shown in Fig. 1.

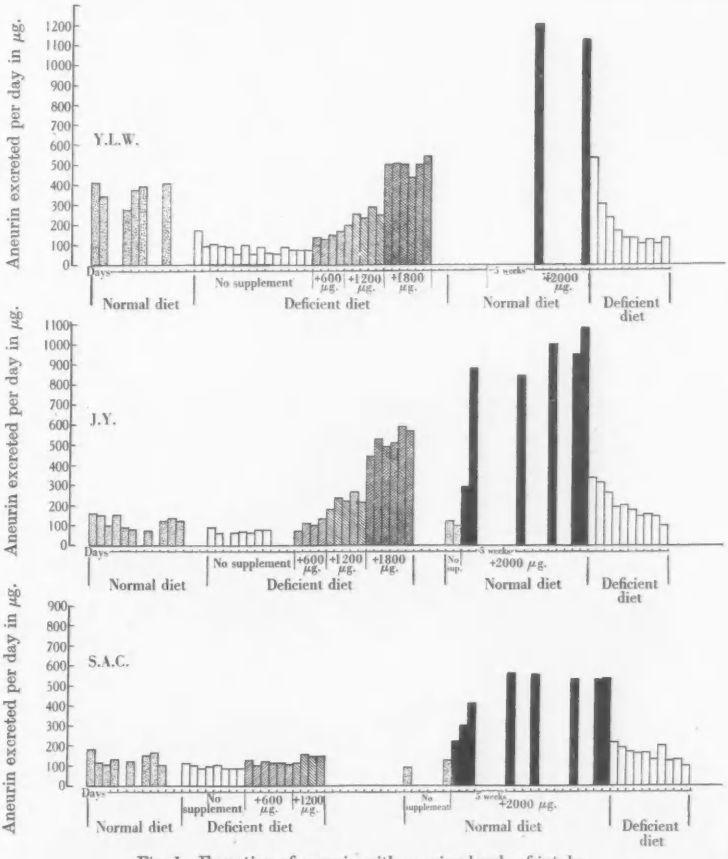


Fig. 1. Excretion of aneurin with varying levels of intake.

It will be seen that the excretion during the period of depletion fell for 5 or 6 days and then reached a value which remained fairly constant until the supplements of aneurin were begun. With each level of supplement, the amount excreted at first increased and then appeared to reach a steady value within 2 or 3 days. The figure also shows the rapidity with which the excretion fell when the intake of aneurin was suddenly decreased after a period of high intake. The supplement of 2 mg. daily on a normal diet was taken for about 7 weeks and then the deficient diet with no supplement was resumed. The excretion fell rapidly to a low level—reaching one-third or less of its previous value in 1 or 2 days—and then continued to fall slowly for the remaining 8 or 9 days of the experiment.

The relation between intake and excretion of aneurin under these conditions is shown in Fig. 2. The aneurin content of the basal diet is taken as 100 μg. The aneurin content of the normal diets was calculated roughly by taking the average excretion on this diet and interpolating on the curve. This gave values of approximately 1200 μg. for S. A. C., 800 μg. for J. Y. and 1600 μg. for

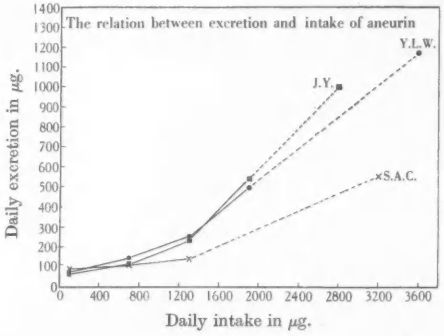


Fig. 2.

Y. L. W. The excretion at each level of intake is taken as the average excretion excluding the first day's value at each level. It will be observed that, with increasing quantities of aneurin ingested, the amount excreted at first increases very little. When the intake reaches some 1200 μg. there is a fairly sudden rise in the amount excreted with increasing intake. The interpretation of these results is discussed below.

(2) *Urinary excretion with intakes of aneurin conforming to reputed requirements*

The reputed daily human requirement of aneurin is 300 I.U. or 900 μg. [League of Nations Commission, 1938]. The excretion at this level of intake was studied with four subjects for a somewhat longer period than at any one level in the previous experiment. As a preliminary, the subjects were depleted for 7 days by taking the basal diet without supplement. The intake of 900 μg. was obtained by a supplement of 800 μg. of aneurin, the basal diet being presumed to contain 100 μg. (see above). By an oversight, however, only 600 μg. were taken for the first 2 days. The supplement of 800 μg. was taken for 7 days. The results of this experiment are shown in Fig. 3. The average daily excretions with the intake of 900 μg., excluding the first day, ranged from 137 to 233 μg. (Table 1).

(3) *The effects of carbohydrate, fat and exercise on aneurin excretion*

The effects of an increase in the amounts of carbohydrate or fat in the diet and of moderate exercise were studied in the four subjects on the basal diet with 800 μg. supplement of aneurin (Fig. 3). Additional carbohydrate was taken as glucose, 100 g. being consumed for 1 day and 200 g. daily for a further 4 days. After an interim period of 3 days on the basal diet with 800 μg. of supplement, 200 g. of fat were added daily to the diet for a period of 4 days. This was taken in the form of butter, made into synthetic cream with a portion of the daily allowance of milk. After a further interim period of 1 day, the subjects were

exercised on a bicycle ergometer for 2 days. The exercise performed by each subject amounted to an equivalent of approximately 500 Cal. each day. The results (Table 1) indicate a slight decrease in excretion with the carbohydrate and with exercise and a less definite decrease with the high fat diet.

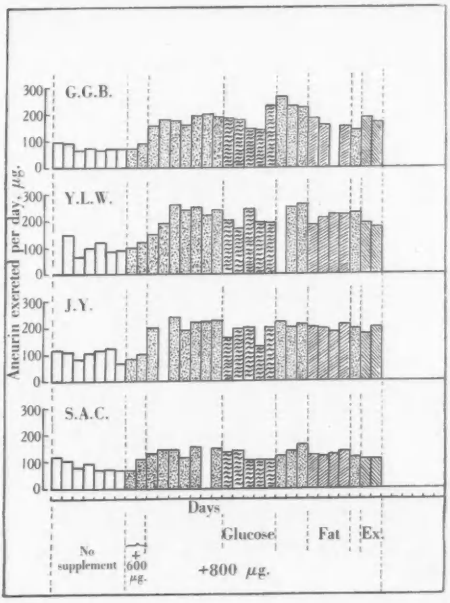


Fig. 3. Excretion of aneurin with intake corresponding to reputed requirement. Effects of carbohydrate, fat and exercise.

Table 1. *Average daily excretion of aneurin with reputed minimum intake (900 μg.)*

	Aneurin excreted per day, μg.				% decrease, average
	S. A. C.	J. Y.	Y. L. W.	G. G. B.	
Basal diet + 800 μg. aneurin	137	219	233	184	—
Basal diet + 800 μg. aneurin + 200 g. glucose daily	109	180	199	174	15
Basal diet + 800 μg. aneurin + 200 g. fat daily	124	198	211	167	10
Basal diet + 800 μg. aneurin + exercise	105	190	180	180	17

(4) *Clinical manifestations of aneurin deficiency*

Although the main object of this experiment was, as we have indicated, to determine the factors concerned in the excretion of the vitamin, a few observations of a clinical nature were carried out during the course of our investigations. They were made with the three subjects who took part in the first experiment, at the beginning and end of the first depletion period of 10–14 days.

We record these mainly because, while our work was nearing completion, a communication appeared in which it was reported that definite subjective and objective manifestations of aneurin deficiency were observed within a few days on a diet deficient in this vitamin [Jolliffe *et al.* 1939]. The subjective symptoms, some of which began to appear 4 days after the diet was started, were said to include anorexia, fatigue, lassitude, precordial pain, dyspnoea on exertion, palpitations, burning of the feet and muscle cramps. On the 5th day, objective signs, such as changes in the electrocardiogram, hyperaesthesia of the legs and tenderness of the calf muscles were observed. It was stated that these symptoms and signs rapidly resolved when large doses of aneurin were taken.

In our experiments, the majority of these manifestations were not observed. Constipation was present in three of the four subjects (excluding J. Y.). Lassitude, fatigue and anorexia were well defined in all subjects. However, these symptoms persisted throughout the periods in which the experimental diet was taken, even when supplements amounting to 1800 μg. were being taken. The symptoms disappeared within 1 or 2 days when our normal diets, which certainly contained less than 1800 μg. of aneurin, were resumed. It is clear therefore that the symptoms were not due to aneurin deficiency, although at present we are unable to account for their appearance. No other symptoms were observed. Objectively, in the few investigations conducted by us we did not encounter the changes described by the American workers. The exercise tolerance was found to be unaltered and the electrocardiogram (in the one case studied) was not affected. The upper limit of audible pitch, which Selfridge [1937] suggests is a sign of aneurin deficiency, was completely unaffected by the lack of the vitamin.

A consideration of the experimental method adopted by Jolliffe and his co-workers makes their results difficult to interpret. During a control period of 5–15 days prior to depletion, each of their subjects took daily supplements of approximately 2·5 mg. of aneurin. The appearance of symptoms of deficiency within so short a time after these large doses seems surprising. More important, however, is the fact that their deficient diet contains relatively large amounts of aneurin. According to these authors their diet contained 474 μg. of aneurin calculated from the tables of Cowgill [1934], or 360 μg. according to Williams & Spies [1938]. These figures seem low when it is observed that their diets included 100 g. beef, 250 g. beet or other vegetable and 200 g. of apple or banana. We have thought it of interest to recalculate the aneurin content of their diet from the tables of Fixsen & Roscoe [1937–8] and are led to assume an aneurin content of some 750–1000 μg. If we accept the latter figures, it would appear that their diet contained approximately the quantity of aneurin accepted as the minimum requirements and probably little below the average normal intake of these subjects. Lastly, the daily excretions of aneurin as measured by Jolliffe and co-workers showed quite large fluctuations, amounting at times to several hundred %, although the diet was constant. The results obtained by us on a steady diet, excluding the first day or two after a change in aneurin intake, were relatively very constant.

Discussion

Variation of excretion with intake

It has been pointed out that, with increasing intake of aneurin, the quantity excreted at first increases only slightly; the curve becomes steeper when the intake exceeds approximately 1200 μg. Extrapolation of the curves to zero intake suggests that some 50–80 μg. of aneurin would still be excreted daily on a

diet completely free from the vitamin. It is of course clear that these values are not equilibrium values; it is certain, for example, that on the deficient diet excretions which are only slightly less than the intake cannot be continued indefinitely. It is known too that a prolonged low intake or decreased absorption of the vitamin, as seen in beriberi or in conditioned deficiencies, is associated with an output approaching zero [e.g. Harris *et al.* 1938; Yudkin, 1938]. But the fact that the quantity of the vitamin excreted at these low intakes approaches a steady value, at any rate for several days, and that small increments in the intake do not produce great changes in the excretion, indicates that, at these low levels, the body is excreting part of its reserve of aneurin. The sharper rise in the excretion values when the intake exceeds some 1200 μg. suggests that above this level the intake is more than sufficient to make up for the daily loss from the body's reserves. The inflexion in the curve is more clearly seen if the excretion is plotted as a percentage of the intake (Fig. 4). In each case, a

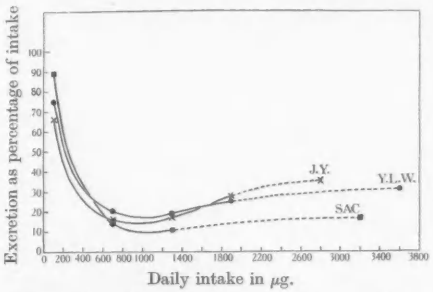


Fig. 4. Excretion of aneurin as percentage of intake.

minimum percentage occurs with an intake between 900 and 1200 μg. Again, the larger percentage excretions below this level are to be explained by more of the excretion being at the expense of reserves as less is ingested. It should be emphasized again that the points on the curve do not represent equilibrium values of excretion.

If this interpretation of our results is correct, then the level of aneurin intake above which the body reserves are no longer being depleted in maintaining the excretion represents the minimal requirement of the body in respect of the vitamin. For the three subjects of this experiment then, the requirements lie between 900 and 1200 μg. or 300–400 I.U., which is in good agreement with the reputed requirement.

In view of the relatively few levels of intake which we have investigated and the difficulties inherent in prolonging or repeating an experiment of this nature with human subjects, we do not wish to attach undue significance to the foregoing suggestions in interpreting our results. At the present juncture we feel that they furnish the most reasonable explanation of the results and, if the interpretation we have put forward is confirmed, they offer an independent method of determining the body's requirements of the vitamin.

The animal experiments of Leong [1937] are of significance in this connexion. With rats fed at different levels of aneurin varying from deficiency to moderately high levels, Leong found that the excretions were related to the intake in a curve very similar to ours for human subjects. A sharp increase in the excretion of aneurin occurred with daily intakes exceeding some 20–25 μg. Estimation of

the vitamin in the tissues showed that intakes below this were associated with a steady depletion of the body stores, whilst on intakes above 20–25 μg. there was an increase in the stores. These results with animals therefore are in agreement with our suggestion that the point of inflexion in the curve relating intake and output of aneurin with human subjects is a measure of their requirements.

The actual values for the excretion of aneurin by the deficient subjects during the course of depletion, viz. 89, 75 and 66 μg. per day, agree well with the provisional conclusion given by Wang & Harris [1939], that a value of less than 30 I.U. (=90 μg.) per day for the "resting level" represents certainly a "sub-average" and probably also a "sub-normal" state of nutrition.

The rapidity of adjustment of excretion to intake

We have seen that any changes due to intake are reflected fairly promptly in the excretion. Even after the daily ingestion of 2 mg. of the vitamin for several weeks, the cessation of this high intake led to some decrease in the quantity excreted within the first 24 hr. The actual level of excretion therefore depends to a great extent on the diet being consumed within the day or two preceding the test. This confirms the conclusion given in earlier papers from this laboratory. It also emphasizes once again the contention that any attempt to estimate the state of past nutrition in respect of aneurin solely by determinations of the current "resting" excretion and without the administration of test doses, is liable to lead to false conclusions.

Individual variation in aneurin excretion

For the three subjects of the first experiment, the quantities of the vitamin excreted at the same low levels of intake were very similar. This suggests that the rate of mobilization of the body's stores of aneurin was similar in these three individuals. With higher intakes the differences between the subjects became more noticeable. For example, with supplements of 800 or 1200 μg. on the deficient diet, or of 2 mg. on a normal diet, subject S. A. C. excreted only about half the quantities excreted by J. Y. On the deficient diet with a supplement of 800 μg., G. G. B. showed an excretion about half-way between that of S. A. C. and those of Y. L. W. and J. Y.

If our suggestions regarding the determination of the body's requirements are correct, these differences in percentage excretion in the three subjects on the higher intakes cannot be due entirely to their different requirements, since, as we have seen, these requirements are all of the same order of magnitude. Possibly the different degrees of excretion represent differences in the rate of absorption, or the kidney threshold, or both. From a practical point of view, the differences in percentage excretion mean that, for subjects whose intake of aneurin is adequate, it is possible only to make an approximate estimate of the vitamin ingested from determinations of the quantity excreted. For example, an excretion of 145 μg. occurred with Y. L. W. on a diet containing 700 μg. of aneurin, whereas the same quantity was excreted by S. A. C. when his diet contained 1300 μg.—nearly twice the amount taken by Y. L. W. It is of course clear that these results with three, or on one level four, individuals cannot be taken as indicating the whole range of normal variation. They do however show that, for the higher levels of intake, this range is fairly wide. On the other hand, daily excretions of 100 μg. or under were in all three cases associated with low intakes—less than about 500 μg.

The assessment of aneurin deficiency

We have seen that a number of workers have attempted to assess the state of nutrition of an individual in respect of aneurin by estimation of the "resting level" of excretion of the vitamin on his ordinary diet. It is evident, however, from the above discussion that this gives only a rough guide. In the first place, owing to the variation existing amongst individuals, the excretion gives only an approximate indication of the intake. Secondly, and more important, the excretion varies so rapidly with the intake that it reflects to a large extent the diet during the day or two preceding the time of urinary excretion. It is clear that other data, such as may be obtained by the administration of "test doses" as suggested by Harris and co-workers [Harris & Leong, 1936; Harris *et al.* 1938; Wang & Harris, 1939], are essential for the detection of the presence and degree of deficiency. As pointed out by Wang & Harris [1939], the deficient case differs from the normal in showing one or more days' delay before the plateau of excretion is reached when the standard test dose is given.

Excretion of aneurin with an intake of the reputed requirement of the vitamin

The quantity of aneurin excreted daily with an intake of 900 μg. (that is, on a deficient diet with a supplement of 800 μg.) varied from 137 to 233 μg. This range is very similar to that of 150–240 μg. found by Wang & Harris [1939], within which lay the excretion values of the majority of normal individuals.

The effect of carbohydrate, fat and exercise

We have seen that quite large increases in the quantity of carbohydrate or of fat in the diet, or moderate increases in the amount of exercise performed, resulted in small decreases in the output of aneurin. The small magnitude of these changes, together with the fact that the observations were conducted only over short periods, do not allow any great significance to be placed on this decrease. However, since moderate deviation from this level of aneurin intake—900 μg.—causes a definite change in the quantity excreted within 1 or 2 days, it is legitimate to conclude that the effect of these decided changes in the diet, and of more moderate increase in exercise, affect very little, if at all, the quantity of aneurin excreted.

Schroeder [1939] claims that a consumption of 250 g. of glucose daily by two patients reduced their aneurin excretion to nil in a fortnight. A study of his results, however, shows that, in both patients, the aneurin excretion did not steadily decrease but fluctuated widely throughout the experiment. On the 10th or 11th day, for example, the excretions of each patient were actually greater than those on the 1st day of the experiment.

Fat has been shown to have an "aneurin-sparing" action in animals, which may be protected from signs of deficiency on a sub-minimal intake of the vitamin if the fat content of the diet is increased. Because of this, it might have been thought that the excretion of aneurin on a steady intake would be increased with a high fat diet. As we have seen, there was in our experiment no appreciable change in the excretion of the vitamin when an additional 200 g. of fat were taken daily. On the other hand, these findings were not unexpected in view of the results of the animal experiments of Kemmerer & Steenbock [1933]. These authors found that a great increase in the amount of fat in the diet did not influence the quantity of aneurin in the tissues of rats, chickens and pigs.

SUMMARY

Three volunteers were placed on a controlled diet deficient in vitamin B_1, graded supplements of the vitamin were administered and the excretion was measured by the method of Wang & Harris. The results may be summarized as follows:

(1) On the basal deficient diet the excretion fell rapidly to a steady value which was maintained for some time.

(2) A constant level of excretion was rapidly attained with each fresh level of intake.

(3) With increasing intake the excretion at first increased only slightly but, when the intake exceeded some 1200 μg., the excretion increased more rapidly. With the reputed requirement of 900 μg. the average excretion (for four subjects) was from 137 to 233 μg.

(4) It is suggested that the excretion at lower levels is at the expense of the body's reserves, and that the level at which the excretion begins rapidly to increase may give an indication of the requirements of the body.

(5) The rapidity with which the excretion adjusts itself to changes in the intake must be taken into account in assessing the "level of nutrition". Additional data, such as the results of test doses, are necessary in order to determine this level.

(6) The percentage excretion at different levels varied somewhat from subject to subject, owing possibly to differences in the rate of absorption or in kidney threshold.

(7) The effects on the excretion of exercise and of variation in the amount of carbohydrate and of fat in the diet have been studied.

We are glad to have this opportunity to express our gratitude to Dr Leslie J. Harris for his invaluable advice and criticism. Our thanks are also due to Mr S. A. Cresswell for his whole-hearted cooperation in these experiments.

REFERENCES

Birch & Harris (1934). *Biochem. J.* **28**, 602.
Cowgill (1934). The Vitamin B Requirements of Man. New Haven.
Fixsen & Roscoe (1937–8). *Nutr. Abstr. Rev.* **7**, 823.
Harris & Leong (1936). *Lancet*, **1**, 866.
—— —— & Ungley (1938). *Lancet*, **1**, 539.
Jansen (1936). *Rec. Trav. chim. Pays-Bas*, **55**, 1046.
Jolliffe, Goodhart, Gennis & Cline (1939). *Amer. J. med. Sci.* **198**, 198.
Kemmerer & Steenbock (1933). *J. biol. Chem.* **103**, 353.
League of Nations Commission (1938). *Bull. Hlth Org.* **7**, 474.
Leong (1937). *Biochem. J.* **31**, 373.
Ritsert (1938). *Klin. Wschr.* **17**, 1397.
Schroeder (1939). *Klin. Wschr.* **18**, 148.
Sciclounoff (1939). *C.R. Soc. biol., Paris*, **131**, 53.
Selfridge (1937). *Ann. Otol., etc., St. Louis*, **46**, 93.
Wang & Harris (1939). *Biochem. J.* **33**, 1356.
—— & Yudkin (1939). *Chem. Ind.* **58**, 1088.
Westenbrink & Goudsmit (1938). *Arch. néerl. Physiol.* **23**, 79.
Williams & Spies (1938). Vitamin B, and its Use in Medicine. New York.
Yudkin (1938). *Lancet*, **2**, 1347.

49. LIVER AMYLASE AND HYPERGLYCAEMIA

By MARGARET LEE and DEREK RICHTER

From the Central Pathological Laboratory, West Park Hospital, Epsom

(*Received 26 January 1940*)

The breakdown of liver glycogen which occurs as one of the chief mechanisms controlling the blood sugar level has generally been ascribed to the liver amylase, but it has recently been suggested that the main path of glycogen breakdown may be through a phosphorylating system involving a phosphorylase which forms hexosephosphate and a phosphatase which hydrolyses the hexosephosphate to glucose [Cori & Cori, 1938].

Recent work has added greatly to our knowledge of the phosphorylating mechanism in the liver [Cori *et al.* 1939; Ostern *et al.* 1939], but if the glycogen breakdown *in vivo* is to be ascribed to this system it would leave the significance of the liver amylase in doubt and there is as yet little reason for attributing this function to the one system rather than to the other.

Cori & Cori [1938] have suggested that the amylase found in the liver may be due to the blood contained in the organ and quote in this connexion an older observation of Davenport [1926] that little amylase is found in the liver after thorough perfusion: but other investigators have concluded that perfusion washes the amylase out of the tissues [Lesser, 1920; Willstätter & Rohdewald, 1936]. There is therefore some doubt as to whether the amylase is really present in the liver tissues or not. The question of the exact localization of the amylase is of special interest since Lesser [1921] concluded that the glycogen and amylase are spatially separated in the liver and that adrenaline acts in producing hyperglycaemia by enabling the glycogen and amylase to come together.

The phosphorylating system has been shown to be reversible, catalysing either the breakdown or the synthesis of glycogen. It is unlikely that there would be more than one system that is able to synthesize glycogen and it is therefore probable that this system is concerned in the synthesis of glycogen *in vivo*; but it is not known that the path of glycogen breakdown *in vivo* is the same as that by which it is synthesized. It is also not known that the mechanism concerned in the normal regulation of the blood sugar is the same as that which comes into action in causing the rapid glycogen breakdown which occurs in the various forms of endogenous hyperglycaemia.

In considering the possible significance of the liver amylase and phosphorylase in relation to hyperglycaemia it is clear that much further information is required before it is possible to reach any final conclusions. In the present investigation particular attention was paid to the significance of the liver amylase and an attempt was made to obtain further evidence on the following questions. (1) Is the amylase present in significant amount in the liver tissue? (2) How are the amylase and phosphorylase distributed between the cells, lymph and blood spaces in the liver? (3) How does the activity of the liver amylase compare with the rate of glycogenolysis which occurs during hyperglycaemia *in vivo*?

Methods

The amylase and phosphorylase activities were determined by incubating tissue brei with glycogen and following the rates of glycogenolysis and phosphorylysis by glycogen and phosphate estimations under suitable conditions.

In order to differentiate between the two enzymes use was made of selective inhibitors and a preliminary study of the action of inhibitors on the enzymes was made for this purpose.

Glycogen. In preliminary experiments it was found that commercial samples of glycogen were not hydrolysed as fast as a specimen prepared in the laboratory. It was thought that this might be due to a modification of the glycogen by the strong alkali used in the manufacture and for the present work the glycogen was therefore prepared by a method that avoided the use of potassium hydroxide or other drastic reagents. The livers of rabbits that had recently been fed with sugar were quickly sliced, dropped into boiling water and boiled for 2 min. The liquid was decanted and the boiled liver minced, ground with sand, returned to the liquid and boiled for 10 min. after adjusting to *p*H 5·3 with acetic acid. The mixture was strained through muslin and the glycogen precipitated with 1·5 vol. of alcohol. After 30 min. the clear solution was decanted and the residue centrifuged. The solid was redissolved in a small volume of 1 % acetic acid, the precipitate removed by centrifuging and the glycogen reprecipitated with alcohol, washed well with acetone and dried.

Glycogen estimation. Glycogen was estimated by the micro-method of Heatley [1935] which was modified only in that larger amounts were taken and the sugar estimations were done by the Hagedorn & Jensen [1923] method. For certain purposes it was found sufficiently accurate and much more rapid to estimate the glycogen by the red colour with iodine. The sample of brei (0·5 ml.) containing glycogen was mixed well with 0·5 ml. 20 % trichloroacetic acid in a centrifuge tube. After centrifuging, 0·5 ml. of the solution was added to 1·0 ml. *N*/20 iodine in 2·5 % KI and the colour compared with standards containing 0·25–1·0 mg. glycogen. Samples giving more colour than the standard range could be diluted and estimated after adding more iodine to bring the iodine concentration to the same as before. The standard solutions kept for about a day.

Phosphate estimation. The presence of glycogen reduces the accuracy of phosphate estimation by the usual molybdate method owing to the turbidity of the final solution used for colorimetric comparison. This was overcome by mixing the specimen (0·5 ml.) in a centrifuge tube with 1·5 ml. alcohol, adding 1 ml. 20 % trichloroacetic acid and keeping overnight before centrifuging and estimating in the usual way as described by Denigès [1921] and Atkins & Wilson [1926].

Determination of amylase and phosphorylase activities. Amylase activities were generally measured by incubating the tissue brei with an excess of glycogen in the presence of a phosphorylase inhibitor and following the rate of glycogen disappearance by glycogen estimations at suitable intervals. In most experiments a total volume of 5 ml. contained 2 ml. liver brei, 0·5 ml. *M*/9 Sørensen phosphate buffer *p*H 7 and sufficient glycogen solution to bring the final concentration with the glycogen already present in the tissue to 1–2 %. As phosphorylase inhibitors 6 % glucose, 0·1 mg./ml. copper added as copper sulphate, or 1 % phloridzin were found to be the most effective. In preparing the liver brei the liver was cooled on ice immediately after perfusion, ground thoroughly with sand in a cooled mortar and mixed with an equal volume of water at 0°. The liver brei was kept on ice and used within 3 hr. of preparation.

In determining phosphorylase activities adenylic acid, hydrogen cyanide [cf. Gill & Lehmann, 1939] and magnesium chloride were added to ensure the activity of the phosphorylating system and sodium fluoride was added to inhibit the decomposition of phosphate esters so that the disappearance of inorganic

phosphate could be taken as a measure of the phosphorylase activity. In most experiments a total volume of 5 ml. contained 2 ml. liver brei, 0·1 ml. adenylic acid (0·1 %), 0·1 ml. $MgCl_2$ solution (1 mg. Mg/ml.), 0·5 ml. $M/2$ HCN prepared by neutralizing KCN with HCl, 0·5 ml. $M/9$ phosphate buffer pH 7, 0·5 ml. $M/2$ NaF and sufficient glycogen solution to bring the concentration to 1–2 %. Tubes containing the mixture were incubated in a water bath at 37°. The rate was not increased by shaking. Samples of 0·5 ml. were taken at the beginning and at various time intervals for phosphate estimation. The initial inorganic phosphate in the tubes, with that coming from the liver brei, was usually equivalent to about 1·5 mg. P. The phosphate uptake due to phosphorylation was generally equivalent to 0·3–0·8 mg. P. The phosphate concentration used in these experiments was chosen as being not too far removed from the physiological concentration. This method of estimating the rate of phosphorylysis of glycogen was subject to error in that even in the presence of $M/20$ NaF the liver breis often showed a slow spontaneous liberation of inorganic phosphate coming possibly from the phospholipins or due to the fact that the phosphatase was not completely inhibited, but the errors incurred in this way were generally small.

The sodium fluoride and other additions used did not appreciably affect the amylase activity; the glycogen breakdown due to the amylase and that due to the phosphorylase were frequently measured simultaneously. Glycogen estimations gave the total rate of glycogenolysis due to both enzymes, while phosphate estimations gave the rate of phosphorylysis. The difference between the total glycogen breakdown and the phosphorylysis then gave the rate of "amylolysis", or hydrolysis by amylase. In calculating the glycogen broken down by phosphorylysis it was assumed that each molecule of phosphate led to the phosphorylysis of one $C_6H_{10}O_5$ unit of glycogen and the formation of one molecule of hexosemonophosphate: the phosphorus uptake figures were therefore multiplied by the factor 5·2 to obtain the phosphorylysis figures.

Preliminary experiments with rabbit liver brei in which 2 % glucose was substituted for glycogen under the conditions described showed no measurable uptake of inorganic phosphate: it was concluded that, in agreement with Ostern *et al.* [1939], no phosphorylation of glucose occurs under these conditions and that the phosphate uptake was entirely due to the phosphorylation of glycogen.

Distribution of amylase in the liver

Perfusion. The possibility of deciding whether the liver amylase is present in the tissues or is mainly confined to the blood spaces in the liver depends on the success with which the blood can be removed by perfusion without causing any loss of amylase from the tissues; considerable attention was therefore paid to the perfusion technique.

Perfusion with distilled water, which was used by Davenport [1926], is liable to cause considerable damage to the tissues owing to the oedema which rapidly occurs and this must hasten the loss of amylase from the tissues. Another source of error is the clotting of the blood in the capillaries through slight damage or chilling of the surface while the liver is being excised. After several different methods had been tried the following was adopted as being the most successful in removing a maximum amount of blood in a minimum of time and with a minimum of damage to the tissues.

Rabbits were anaesthetized with nembutal (about 25 mg./kg. intravenously). The abdominal cavity was opened and a loose ligature rapidly put in position round the portal vein. A hypodermic needle delivering normal saline at 37°

and from a height of 40 cm. was then inserted into the portal vein, the ligature tied round the needle and the vena cava and aorta cut to allow the blood to escape freely. The removal of blood from the liver was hastened by gentle massage. After about 7 min. of rapid perfusion, when the perfusate appeared free from blood, the liver was taken out and immediately cooled on ice.

The efficiency of the perfusion was frequently checked by centrifuging a suspension made by shaking the minced liver in saline: any red blood corpuscles which might be present then formed a fine layer on the surface which enabled the presence of a very small amount of blood to be detected. It was estimated that in none of the experiments was the residual blood as much as 1 % of that originally present or 0·3 % of the weight of the liver; it could therefore be taken that at least 99 % of the blood amylase was removed in this way.

Glycogenolytic activity of perfused liver

The activity of the brei prepared from the perfused rabbit livers in breaking down glycogen was tested under the conditions described. The activity varied greatly from one animal to another: frequently the activity was very high and corresponded to the glycogenolysis of 20–50 mg. glycogen per g. fresh liver per hr., but in many experiments the glycogenolysis did not exceed 1 mg. glycogen per g. liver per hr. The rate of glycogenolysis under these conditions remained constant for several hours or until nearly all the glycogen had disappeared.

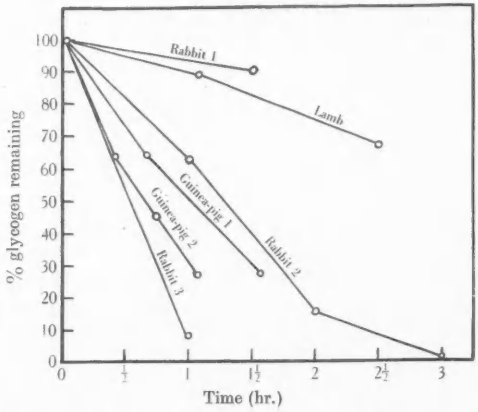


Fig. 1. Glycogen breakdown by liver brei. Guinea-pig 1 and rabbit 2 livers perfused; guinea-pig 2, rabbit 1 and 3 and lamb livers not perfused. Initial glycogen concentration 1 %.

At this stage it was not known whether the glycogenolysis in the liver brei under these conditions was due mainly to an amylase or to the phosphorylase. Experiments were therefore carried out to test this by means of specific amylase and phosphorylase inhibitors.

Amylases are known to be strongly inhibited by maltose, which is a product of their action on glycogen, and phosphorylases are inhibited by phloridzin. Gill & Lehmann [1939] have recently made an extensive study of the action of inhibitors on the muscle phosphorylase. Their observations on the inhibition of muscle phosphorylase by glucose, maltose and copper were confirmed. The

actions of 6% glucose, 12% maltose, 1% phloridzin and 0·1 mg./ml. copper on (*a*) pancreatic amylase, (*b*) blood amylase and (*c*) muscle phosphorylase were then compared with their effects on (*d*) the total glycogenolysis and (*e*) the phosphorylysis measured simultaneously under the same conditions in freshly perfused rabbit liver brei (Table 1).

Table 1. *Percentage inhibitions by maltose, glucose, phloridzin and copper*

Inhibitor	Pancreatic amylase	Blood amylase	Muscle phosphorylase	Liver brei: Total glycogenolysis	Liver brei: Phosphorylysis
6% glucose	0	2	100; 73; 100	5; 0; 11	82; 100; 41
12% maltose	85	33	50; 100; 67	66; 45	60
1% phloridzin	2	8	50	13; 0; 8	56; 47; 24
0·1 mg./ml. copper	8	3	100; 100; 78	0; 0; 0	29; 19; 26

The liver brei was prepared from rabbit liver which gave a breakdown of 15–25 mg. glycogen per g. liver per hr. The copper was added as $CuSO_4$.

The pancreatic amylase was prepared from sheep pancreas and purified by the method of Hanes & Cattle [1938]. Ox blood was used as a source of blood amylase. Muscle phosphorylase was used in the form of an extract made from rabbit muscle as described by Meyerhof [1926]. The enzyme solutions were diluted so as to give a glycogen breakdown of about 50% in 2 hr. with an initial concentration of 1–2% glycogen. The amylase experiments were done in the presence of 10 mg./ml. NaCl and *M*/30 phosphate buffer *p*H 7. The conditions of the experiments with muscle extract and liver brei were as described above.

Maltose inhibited the phosphorylase as well as the amylase and so failed to differentiate between them but the inhibitions observed with glucose, copper and phloridzin gave clear evidence that an amylase was present in the liver brei and was mainly responsible for the glycogenolysis observed under these conditions. This was confirmed by the figures giving the phosphorylysis calculated from measurements of the phosphate uptake (Table 2).

Table 2. *Glycogenolysis and phosphorylysis in perfused rabbit liver brei*

Exp. no.	Incubation hr.	Total glycogenolysis mg./g./hr.	Phosphorylysis mg./g./hr.
1	$2\frac{1}{4}$	10·7	1·07
2	2	15·0	0·98
3	$1\frac{1}{4}$	25·6	3·94
4	$1\frac{3}{4}$	3·4	0·86
5	$\frac{1}{2}$	24·0	4·23
6	$2\frac{1}{4}$	26·0	1·06

Conditions as described for measuring total glycogenolysis and phosphorylysis simultaneously.

The figures for total glycogenolysis and phosphorylysis represent mg. glycogen broken down per g. fresh liver per hr. The phosphorylysis was always much less than the total glycogenolysis, indicating again that most of the glycogenolysis under these conditions was due to the amylase.

The rates of total glycogenolysis and phosphorylysis were also measured in the same way in the brei prepared from unperfused rabbit liver. The blood was roughly removed by pressing out on filter paper.

The results with unperfused liver (Table 3) were very similar to those obtained with perfused liver and showed that the presence or absence of blood did not produce any very striking difference such as might have been expected if the

Table 3. *Glycogenolysis and phosphorylysis in unperfused rabbit livers*

Exp. no.	Incubation hr.	Total glycogenolysis mg./g./hr.	Phosphorylysis mg./g./hr.
1	$1\frac{3}{4}$	21·6	2·01
2	2	4·8	1·04
3	1	55·0	2·50
4	$1\frac{1}{2}$	5·3	2·24
5	1	24·0	2·94
6	$1\frac{3}{4}$	21·6	2·01
7	1	14·4	1·00

Conditions as described for measuring total glycogenolysis and phosphorylysis simultaneously.

blood amylase were an important factor. There was also no evidence of any loss of amylase or phosphorylase activity during the perfusion.

In other experiments with fresh unperfused livers from other species the absence of any considerable inhibition of glycogenolysis by glucose, phloridzin or copper again showed that under these conditions most of the glycogen breakdown in the liver brei was due to "amylolysis" rather than to phosphorylysis.

Animal	Incubation hr.	Total glycogenolysis mg./g./hr.	Percentage inhibition		
			Glucose	Copper	Phloridzin
Guinea-pig	2	12·8	16	0	0
Sheep	2	3·2	0	0	—
Lamb	2	3·2	0	—	0

The sheep and lamb livers were used within 45 min. of slaughtering. The inhibitor concentrations and other conditions were the same as described for rabbit liver.

Distribution of amylase between tissue and blood

The foregoing experiments gave evidence of the existence of an active amylase in the liver tissue. The similar results obtained with perfused and unperfused livers made it improbable that the amylase found in the liver could be attributed to traces of blood remaining in the liver after the perfusion, but it was desired to have more definite evidence on this point and in a number of experiments the amylase contents of the blood and perfused liver from the same animal were tested under the same conditions and at the same time.

Table 4. *Amylase activities of liver and blood from the same animal*

Exp. no.	Animal	Incubation time min.	Additions	Glycogenolysis		Remarks
				Liver mg./g./hr.	Blood mg./g./hr.	
1	Rabbit	65	—	9·2	10·1	—
2	,,	65	—	55·0	50·0	Not perfused
	,,	65	6% glucose	52·0	50·0	,,
3	,,	105	—	3·4	5·7	—
	,,	105	1% phloridzin	3·4	5·7	—
4	,,	150	—	10·7	9·8	—
	,,	150	6% glucose	9·8	9·8	—
5	Guinea-pig	60	—	42	99	—
	,,	60	1% phloridzin	45	99	—
6	,,	65	—	23·4	27·6	Not perfused

The livers were perfused except where stated. Conditions as described.

In five out of six experiments (Table 4) the amylase activities of the blood and liver, expressed as mg. glycogen broken down per g. per hr., were very similar.

In vivo about 30 % of the liver weight is due to blood, but after excision and pressing out this figure may be reduced to 5–10 %. The amount of blood remaining after perfusion was estimated as less than 0·3 %. It was therefore evident that the amylase activity of the blood left in the organ could not possibly account for the activity found in the liver. It was clear that the amylase must be present in the liver tissue and that the concentration in the tissue was similar to that in the blood.

Similar experiments in which the distribution of the phosphorylase was tested showed that it was present exclusively in the tissues: no phosphorylase activity was found in the blood.

Distribution of amylase between cells and intercellular fluid

In stained sections prepared from cat, rabbit and pig livers after fixation in formalin the volume of the intercellular or lymph spaces between the cells and sinusoidal membranes always appeared to be small; but this may be due to shrinkage of the membranes on to the cells in the fixing and staining. There is evidence that the ratio of intercellular space to cell volume may vary considerably *in vivo* under different physiological conditions and the marked change in liver volume produced by adrenaline suggested that such a change might be related to the hyperglycaemic action and made it appear desirable to try to obtain evidence as to whether the amylase is normally present in the cells or is present only in the intercellular fluid.

In ordinary liver brei prepared by grinding in a mortar the texture is generally uneven, and microscopic examination after staining with toluidine blue shows a considerable proportion of damaged cells and cell fragments. After several different methods had been tried it was found that by gently pressing liver slices of about 1–2 mm. thickness first through a coarse net of 25 holes per sq. cm. and then through a fine net of 170 holes per sq. cm. and suspending in normal saline a cell suspension could be obtained consisting mainly of single cells and small clusters of cells in which very few damaged cells or cell fragments could be found. The cells quickly separated out on centrifuging and in this way a rough separation of the cells from the intercellular fluid was obtained. The suspending fluid may be expected to contain most of the enzymes present in the lymph and intercellular fluid as well as any enzymes washed out of the cells.

For testing the amylase activities the liver which was cooled on ice immediately after perfusion was sliced as rapidly as possible, pressed through net into a cooled centrifuge tube, shaken rapidly with an equal volume of normal saline and the suspension immediately centrifuged at high speed. The time taken from starting to cut the slices to centrifuging was about 2 min. The separated cells were ground with sand and made into a brei with an equal volume of water. The amylase activities of the cells and suspending solution were then tested in the usual way. In most experiments 6 % glucose or 1 % phloridzin was added to inhibit the phosphorylase, but this did not appear to be necessary as the phosphorylase was relatively inactive under the conditions used, which were not optimal for showing phosphorylase activity. Two experiments are given in which 6 % gum acacia in normal saline was used as a suspending medium for the cells (Table 5). Control experiments showed that the gum acacia did not inhibit the amylase.

Table 5. *Percentage distribution of amylase between liver cells and suspending fluid*

Exp. no.	Animal	Glycogenolytic activity mg./g./hr.	Additions	Incubation time min.	Distribution of amylase (%) Cells	Fluid	Remarks
1	Guinea-pig	27·2	—	60	66	34	—
	,,	27·2	1% phloridzin	60	67	33	—
2	,,	24	—	95	53	47	Cells washed
3	,,	10·2	1% phloridzin	60	72	28	Cells washed
4	Rabbit	4·8	—	60	52	48	—
5	,,	24·0	6% glucose	60	54	46	Not perfused
6	,,	25·6	—	75	85	15	Gum saline
7	Lamb	3·2	6% glucose	150	66	34	Gum saline; not perfused

In exps. 3 and 4 the cells were washed by rapidly resuspending in saline and recentrifuging before estimating the amylase activity. Conditions as described.

In every experiment more amylase was found in the cells than in the suspending fluid containing the lymph and intercellular fluid belonging to them. The activity of the suspending fluid was probably due in part to amylase washed out of the cells, as by washing the cells at the ordinary temperature the amylase was soon removed from them; but in two experiments in which the cells were rapidly resuspended in saline at 0° and centrifuged off again the amount of amylase left in the cells was still greater than that in the original suspending fluid.

It was concluded that the amylase occurs to a greater extent inside the liver cells, or closely attached to them, than in the intercellular fluid or lymph.

Distribution of phosphorylase

Similar experiments carried out to test the distribution of the phosphorylase indicated that it is also mainly present in the cells. In these experiments sodium fluoride was added and the phosphorylase activity was measured by the inorganic phosphate uptake under the conditions described.

Exp. no.	Animal	Incubation time hr.	Phosphorylysis mg./g./hr.	Distribution of phosphorylase (%) Cells	Fluid	Remarks
1	Rabbit	1	2·9	62	38	—
2	,,	$\frac{1}{2}$	6·3	72	28	—
3	,,	$1\frac{1}{4}$	3·9	63	37	Gum saline

Effect of drying with acetone on glycogenolytic system

Many previous investigators have used "acetone powders" made by grinding the liver with acetone in studying the properties of liver "glycogenase". Hodgson [1936] claimed that the acetone powder prepared from liver is free from blood amylase since the blood amylase is destroyed in this process. It appeared of interest to test whether a real separation of the enzymes could be obtained in this way.

A dry acetone powder was prepared from rabbit liver as described by Hodgson. The powder (1 g. in 5 ml.) was then incubated with glycogen and the total glycogen

breakdown and phosphorylysis measured under the conditions described for the fresh liver brei.

Time of incubation hr.	Total glycogenolysis mg./g./hr.	Phosphorylysis mg./g./hr.
1½	0·65	0·43

The glycogenolysis and phosphorylysis figures, which give the activities per g. of the original fresh liver, indicated that the preparation contained both amylase and phosphorylase with relatively more phosphorylase than is usually present in fresh liver; but the activity of the preparation was low and suggested a considerable loss during the preparation. Contrary to Hodgson, an acetone powder prepared in the same way from blood showed considerable amylase activity.

Amylase in muscle and other organs

Gill & Lehmann [1939] have suggested that the amylase found in muscle may provide an alternative path of glycogen breakdown to the normal phosphorylysis. Parnas [1937] on the other hand was unable to find any amylase in muscle, which suggests that, as in liver, the amount of amylase present may be very variable.

We were able to confirm the presence of amylase in muscle extracts prepared by the method of Meyerhof [1926] from the leg muscles of rabbits which had been perfused *in vivo* with saline through the descending aorta:

Exp. no.	Incubation time hr.	Total glycogenolysis mg./g./hr.	Phosphorylysis mg./g./hr.	Remarks
1	1½	12·0	1·3	Perfused
2	3	6·7	0·53	Not perfused
3	3	5·3	0·74	Perfused

Under the conditions of these experiments, which were the same as those described for measuring glycogenolysis and phosphorylysis simultaneously in liver, the phosphorylysis was much too low to account for the total glycogenolysis. It should perhaps be emphasized that the low phosphorylysis rates shown in these experiments, as in those with liver, do not mean that active phosphorylases were not present in the tissues. The phosphorylysis rates appear low because the conditions were chosen as being optimal for the amylase and were unsuitable for optimal phosphorylase activity. The phosphorylases are very labile and soon destroyed under these conditions, but by using short incubation periods and higher phosphate concentrations high rates of phosphorylysis of over 15 mg. per g. per hr. were easily obtained with both liver and muscle [Lee & Richter, 1940].

In a number of other experiments the rates of glycogenolysis were measured in brei prepared from other animal organs. The conditions were as described for measuring amylase activity; under these conditions phosphorylysis was always very small and the glycogen breakdown was mainly due to amylolysis:

Organ	Incubation time hr.	Glycogenolysis mg./g./hr.	Organ	Incubation time hr.	Glycogenolysis mg./g./hr.
Ox blood	¾	31·8	Lamb brain	2	0
Rabbit blood	2½	34·5	Lamb pancreas	¼	578,000
Lamb blood	2¼	0	Lamb liver	2¾	4·3

It appeared that evidence of the ability of the amylase to diffuse through membranes might be obtained by testing if the blood amylase is able to pass through the haemotoencephalic barrier into the cerebrospinal fluid. Marchionini [1935] has described the presence of amylase in the C.S.F. and in order to confirm this three specimens of C.S.F. which had been sent to this laboratory for Wassermann tests and found to be normal were incubated (*a*) with starch and (*b*) with glycogen at 37°. Each tube contained 1 ml. *M*/15 phosphate buffer *p*H 7·4, 1 ml. normal saline, 0·2 ml. 1 % starch or glycogen, 1 ml. C.S.F. or water, 1 ml. 50 % glucose or water and 0·02 ml. toluene. When tested with iodine a slow disappearance of starch or glycogen, which was not inhibited by glucose, was observed in the tubes containing C.S.F.

Discussion

Origin of liver amylase. Evidence has been obtained that an amylase occurs not only in the blood contained in the liver but also in the liver tissue. Previous investigators have generally concluded that the amylase activity is much greater in the blood than in the liver [Macleod, 1926; Davenport, 1926] but this may be due to the loss of liver amylase during the perfusion or through incomplete extraction. In five out of six experiments in which the amylase activities in the liver and blood from the same animal were measured at the same time the amylase activities, expressed as mg. glycogen broken down per g. tissue per hr., showed a closed agreement (Table 4).

Macleod & Pearce [1909], who compared the blood and liver amylase activities in a number of animals, found that the blood was generally, but not always, the more active and they failed to find any relation between them except for noting that in three experiments in the pig, rabbit and sheep the blood and liver amylase activities appeared to run parallel. The failure of Macleod & Pearce to observe a more definite relationship between the blood and liver amylase activities may be due to their generally omitting to make the comparison at the same *p*H, for in four experiments carried out for another purpose in which buffer was added [Macleod & Pearce, 1911] their figures, recalculated in terms of mg. glycogen broken down per g. per hr., agree with the present observations in indicating a similarity in the blood and liver activities:

Exp. no.	Glycogenolysis in blood mg./g./hr.	Glycogenolysis in liver mg./g./hr.
1	48·6	41·8
2	45·0	53·0
3	34·7	16·1
4	27·2	27·6

It is generally accepted that the blood amylase is derived mainly from the pancreas, since the blood amylase activity is reduced by pancreatectomy and is considerably affected in disease of the pancreas. If, as has been widely believed, the liver amylase is specially synthesized in the cells and has no connexion with the amylase in the blood, there would be no reason to expect any relation between them and the ratio of their activities might lie almost anywhere within a wide range. The observation that in spite of the great individual variation in their absolute amounts the amylase concentrations in the blood and liver so frequently agree can hardly be due to chance. It is concluded that the two are probably related and that the liver amylase is derived from, and normally in equilibrium with, that in the blood.

If the amylase concentration in the liver is maintained equal to that in the blood simply by diffusion from the blood, the amylase must be able to diffuse into the liver cells which occupy the greater part of the liver volume. Independent evidence that the amylase occurs in the cells was obtained by estimating the distribution of amylase between the cells and intercellular fluid. It may be questioned whether it is possible for an enzyme to pass through the cell membranes into the cells, but the amylase is known to diffuse from the pancreas into the blood and through the membranes of the kidney into the urine: it is also found in the lymph and can pass through the haematoencephalic barrier into the cerebrospinal fluid. This gives evidence that the amylase is particularly easily diffusible. The amylase is known to diffuse rapidly out of the tissue when liver slices are suspended in normal saline [Willstätter & Rohdewald, 1936] and there is evidence that it may diffuse out of the liver on perfusion. It does not therefore appear impossible that the amylase may diffuse at a significant rate in the reverse direction so that in the course of time the concentration in the cells *in vivo* may approximate to that in the blood.

Function of liver amylase. The activity of the amylase in the rabbit liver was frequently very low and generally too low to account for the rate of glycogenolysis occurring in the more intense forms of hyperglycaemia *in vivo* [Lee & Richter, 1940]; but in many animals the activity of the liver amylase was sufficient to account for a breakdown of 20–50 mg. glycogen per g. per hr.

It is probable however that such high activities do not occur *in vivo* since it has been shown previously that the liver amylase is activated by chloride ions and requires a considerable chloride concentration for optimal activity. The activity rapidly falls off at chloride concentrations below 35 mg. Cl/100 ml. [Eadie, 1927]. Bang [1913] obtained experimental evidence that the chloride concentration in the cells in the frog's liver is well below the optimal for amylase activity since, after perfusing the liver with isotonic sodium sulphate or sugar solution, the amylase was strongly activated by adding sodium chloride. Evidence has recently been accumulating that the concentration of free chloride ions inside the cells in mammalian muscle, liver and other organs is extremely low and it is frequently accepted that practically the whole of the chloride in the tissues is extracellular [Fenn, 1936; Bourdillon, 1937; Amberson *et al.* 1938]. The liver glycogen is known to be confined to the cells, apart from the relatively small amounts present in the blood and in the leucocytes, but there is reason to believe that the amylase present in the cells may be inactive, or much less active than the amylase activities which have been described would indicate, owing to the absence of a sufficient concentration of free chloride ions in the cells.

It has been shown elsewhere that the activity of the liver phosphorylase, when examined under suitable conditions, is very high and sufficient to account for the most intense forms of hyperglycaemia known *in vivo* [Ostern *et al.* 1939; Lee & Richter, 1940]; there is therefore no need to assume that the amylase is concerned in any normal hyperglycaemic mechanism. The blood amylase might possibly have a function in the detoxication of polysaccharides of bacterial origin, but we have no evidence of this and it can hardly be a function that is general to all the mammalia since Macleod & Pearce [1909] found, and we have confirmed, that in the sheep the amylase concentrations in the blood and tissues are always very small and frequently none can be found at all in the blood or liver in the lamb.

If the distribution of amylase is considered in the animal as a whole it is evident that by far the highest amylase concentrations are in the pancreas and salivary glands. In one experiment in the sheep the amylase activity in the

pancreas was found to be 578,000 compared with 3·4 in the liver. It is clear that even the highest activities found in the blood, liver and other organs were only of the order of 1/10,000 of the amylase activity of the pancreas and therefore small in comparison.

The amylases of the pancreas and salivary glands have a clearly defined function in the digestion of starchy foods in the stomach and intestine, but it is difficult to ascribe any important function in carbohydrate metabolism to the relatively small and very variable amounts of amylase that are found in the blood and other organs. There would appear to be no need to regard the small amounts of amylase found in the liver and other organs as consisting of anything more than traces of the very active pancreatic and salivary amylases which have diffused into the blood and throughout the system.

Summary

1. The amylase activity in the liver in the rabbit is very variable. The activity may be high enough to cause a breakdown of 50 mg. glycogen per g. liver per hr.

2. Methods are described for distinguishing between the glycogen breakdown due to the amylase and that due to phosphorylysis.

3. In five out of six experiments the amylase concentration in the liver was similar to that in the blood.

4. The liver amylase is not confined to the blood or lymph spaces, but is present inside or closely bound to the cells.

5. The glycogen phosphorylase also occurs inside or closely bound to the cells.

6. The phosphorylase is present in relatively larger amount than the amylase in the dry powder obtained by treating liver with acetone.

7. It is concluded that the amylase found in the liver is derived from the blood and is not concerned in any normal hyperglycaemic mechanism.

The authors wish to thank Dr H. Lehmann for useful advice and the Rockefeller Foundation for supporting this investigation.

REFERENCES

Amberson, Nash, Mulder & Binns (1938). *Amer. J. Physiol.* **122**, 224.
Atkins & Wilson (1926). *Biochem. J.* **20**, 1223.
Bang (1913). *Biochem. Z.* **49**, 40.
Bourdillon (1937). *Amer. J. Physiol.* **126**, 411.
Cori & Cori (1938). *Proc. Soc. exp. biol., N.Y.*, **39**, 337.
—— —— & Schmidt (1939). *J. biol. Chem.* **129**, 629.
Davenport (1926). *J. biol. Chem.* **70**, 625.
Denigès (1921). *C.R. Soc. Biol., Paris*, **17**, 1974.
Eadie (1927). *Biochem. J.* **27**, 314.
Fenn (1936). *Physiol. Rev.* **16**, 454.
Gill & Lehmann (1939). *Biochem. J.* **33**, 1151.
Hagedorn & Jensen (1923). *Biochem. Z.* **135**, 48.
Hanes & Cattle (1938). *Proc. roy. Soc.* B, **125**, 412.
Heatley (1935). *Biochem. J.* **29**, 2568.
Hodgson (1936). *Biochem. J.* **30**, 542.
Lee & Richter (1940). In the Press.
Lesser (1920). *Biochem. Z.* **102**, 304.
—— (1921). *Biochem. Z.* **119**, 108.
Macleod (1926). Carbohydrate Metabolism and Insulin. London: Longmans, Green & Co.
—— & Pearce (1909). *Amer. J. Physiol.* **25**, 255.
—— —— (1911). *Amer. J. Physiol.* **28**, 403.
Marchionini (1935). *Dtsch. Z. Nervenheilk.* **138**, 83.
Meyerhof (1926). *Biochem. Z.* **178**, 395.
Ostern, Herbert & Holmes (1939). *Biochem. J.* **33**, 1858.
Parnas (1937). *Ergebn. Enzymforsch.* **6**, 57.
Willstätter & Rohdewald (1936). *Enzymologia*, **1**, 213.

50. AN IMPROVED CELL FOR MEASUREMENTS OF OXIDATION-REDUCTION POTENTIAL

By LEON GROTIUS ZERFAS and MALCOLM DIXON

From the Biochemical Laboratory, Cambridge

(*Received 19 January 1940*)

For the work described in the following paper [Dixon & Zerfas, 1940] we required an apparatus for conveniently making measurements of oxidation-reduction potential during certain enzyme reactions. This apparatus had to fulfil the following requirements: (*a*) It must contain some arrangement for adding several reagents successively to the enzyme solution without opening the apparatus, introducing any traces of O_2 or interrupting the readings. (*b*) It must contain provision for removing all traces of O_2. (*c*) There must be no possibility of contamination of the solution with traces of the reagents used in previous experiments. The latter point was of great importance, because the possibility that some coenzyme might have been carried over from a previous experiment would have destroyed the evidential value of the results.

A number of cells have been designed by previous workers, among which we may mention those of Lehmann [1930] (two types), Wurmser & Geloso [1931], Dixon & Kodama[1] [Tsukano, 1932] and Borsook & Schott [1931]. None of the existing cells fulfilled the conditions given above and we therefore had to develop a special cell for our purpose. This cell proved very satisfactory and we give here a detailed description of it in the belief that it may be useful for general work.

In general freedom from O_2 may be secured either by passing a stream of O_2-free N_2 or by evacuation. The first method is not very convenient, as it involves setting up apparatus for the supply in fairly considerable quantity of N_2 from which every trace of O_2 has been removed. Unless this is done very efficiently, appreciable quantities of O_2 may be passed into the solution in the course of an hour or two, along with the N_2. The vacuum method is much more convenient, and all the cells mentioned above depend on this principle. It must be pointed out, however, that it is not sufficient merely to exhaust the apparatus on a water-pump and to close it off immediately. Under these conditions the O_2 dissolved in the liquid is not removed and remains in the apparatus throughout the experiment. It is best to wash out the cell once or twice with N_2 before the final evacuation, and even then some provision should be made (e.g. a side-bulb containing alkaline pyrogallol) for the removal of any remaining O_2 traces. Only one of the above-mentioned cells (that of Dixon & Kodama) contains such provision.

The usual way of adding a reagent to an electrode vessel is from a burette leading through the stopper of the vessel. This involves the protection of the upper end of the burette from the air and the careful removal of dissolved O_2 from the solution in the burette. When several different solutions have to be added this method becomes very troublesome, and it is much better to have some arrangement inside the cell itself for adding the different solutions. Only

[1] The original form differed from Tsukano's illustration by the inclusion of a built-in agar salt bridge and an extra side-bulb for alkaline pyrogallol.

one of the above-mentioned cells (the "large" electrode vessel of Lehmann [1930, p. 243]) contains such provision. The upper part of this cell contains four cups mounted on pivots in such a way that they can be inverted by a movable arm, spilling their contents into the solution in the lower part of the electrode vessel. We have adopted this arrangement in principle, but using an electrical method which is very simple in construction and avoids an extra joint with possibility of leakage.

It is necessary to have some method of making contact between the solution in the electrode vessel and the standard half-cell which forms the second electrode of the system. The usual way of effecting this is by means of an agar-KCl bridge, and all the cells except one (that of Wurmser & Geloso) have such a salt bridge built in as a permanent part of the apparatus. This is undesirable for the following reason. During an experiment substances diffuse from the solution into the end of the salt bridge through the agar. During the next experiment these substances contaminate the solution by diffusing out from the salt bridge. Filling the vessel with KCl solution and allowing it to stand between experiments reduces, but does not eliminate, the effect, as some substances may diffuse into the bridge for some distance. The amounts involved are usually small, but we have found that they are important in some cases.

The apparatus of Wurmser & Geloso does not involve a salt bridge, but makes use of the conductivity of the film of water on the inner surface of the glass. This conductivity is of course extremely small compared with the salt bridge and it is necessary to use some form of thermionic potentiometer.

In order to overcome the difficulty we have arranged the standard half-cell so that a separate salt bridge is used; the bridges are easily prepared in quantities of fifty or more, and each bridge is used once only and then discarded. By this means all risk of contamination is avoided.

The apparatus is shown in Fig. 1. The main vessel carries on one side the side-bulb S for the pyrogallol and on the other the part H for the standard half-cell. The lower part, in which the solution to be studied is placed, is blown out as shown to form a bulb about 1 in. in diameter, in order to facilitate mixing of the solution. A gentle oscillation of the apparatus is sufficient to keep the liquid round the electrode thoroughly mixed. The vessel is closed at the top by a well-fitting rubber bung,[1] through which pass (*a*) the glass tube carrying the main electrode E, (*b*) a tube with a well-ground tap T for evacuating the vessel, (*c*) a wide tube, closed at the lower end, through which are sealed five platinum wires. The part H is closed with another rubber stopper, which carries the second electrode for the half-cell. The upper rims of the vessel are expanded so as to form water-seals round the stoppers, and thick liquid paraffin is run round all the tubes where they pass through the stoppers in order to prevent all possibility of leaks. The tube carrying the main electrode is bent as shown so that the electrode is brought near the central axis. The electrodes are both of bright platinum and contact is made through mercury in the tubes with the leads to the potentiometer (not shown) which are dipped into the open ends of the tubes. The salt bridges B are made of glass tubing, 2·5 mm. internal diameter, and are bent to fit a template. Their shape should be carefully adjusted so as to allow them to be readily inserted with forceps through the passage from the main vessel into the part H, and at the same time to allow their ends to dip well into the solutions in both parts of the vessel. Their preparation is described below.

[1] No doubt the apparatus could be improved by an all-glass construction with ground joints, but we have found the rubber stoppers quite satisfactory and the cell as described is easy to construct.

The central tube with its wires is for adding the reagents and the arrangement operates as shown in the detailed view (Fig. 2). The parts of the wires passing

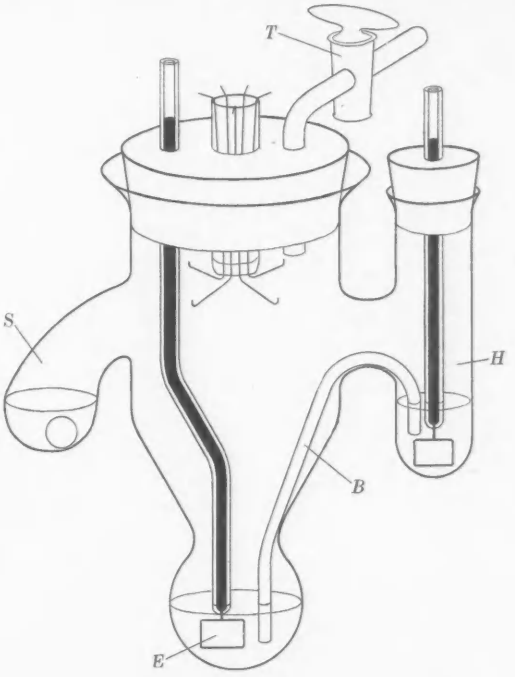


Fig. 1.

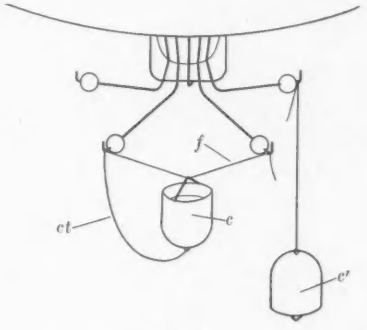


Fig. 2.

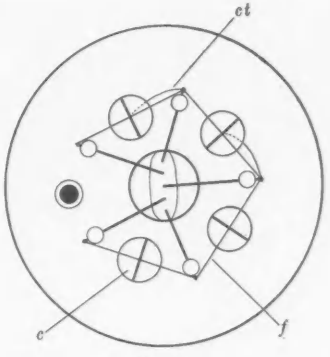


Fig. 3.

up through the wide tube are insulated from one another by lengths of narrow glass tubing slipped over them. A differently coloured bead is placed on the

upper end of each wire for identification, and a few mm. of bare wire should project so that connexion to an accumulator can be made through leads carrying "crocodile clips". The lower ends are bent radially outwards horizontally like the spokes of a wheel. The end of each wire carries a bead corresponding in colour with that on its other end, and is bent upwards to form a small hook. Each reagent to be added is placed in a small glass cup (*c*) holding about 1 ml., which has a handle formed of a piece of platinum wire fused on across its open end as shown. By means of this it is hung from a length of fuse wire (*f*) fastened across the ends of two of the platinum wires. Thus on connecting the upper ends of these two wires to a battery a current passes through the fuse wire, which melts and parts in the centre so that the cup falls. It does not however fall into the solution in the electrode vessel. A small loop of platinum wire is fused on to the bottom of each cup and to this is fastened a short length of cotton thread (*ct*) terminating in another small platinum loop. The latter is hung over the end of one of the supporting wires, so that when the cup falls it hangs in an inverted position as shown at *c'*, delivering its contents into the solution below. The cups are coated inside with a thin layer of high-melting paraffin wax and this ensures that the delivery of the contents is complete.

As there are five supporting wires four cups can be used, as shown in plan in Fig. 3, and any cup can be dropped at any time independently of the others by connecting the correct pair of wires to the battery. The wires must of course be stout enough to take the weight of the cups without bending. They are bent out so that the gap in the ring comes where the electrode stem passes up, as shown in the plan.

The arrangement is assembled as follows. The rubber bung with its attachments is held in a convenient stand. A piece of fuse wire (fine tinned copper wire rated at 3 amp.) of sufficient length is attached to the end of one of the supporting wires by winding it round several times with forceps. It is then threaded through the handle of one of the cups and attached to the next supporting wire in the same way. Another cup is then threaded on and the fuse wire attached to the next supporting wire and so on until all the cups are hanging. The colours of the corresponding beads are noted down at the same time. It is important that the wires should be free from grease as otherwise a bad contact may possibly give trouble. The loops on the ends of the threads are then dropped over the hooks on the adjacent supporting wires and the cups are examined to make sure that they hang freely in the centre of their length of fuse wire. This part of the apparatus is then ready. The lengths of thread should be long enough for the cups to hang upright, but short enough to keep them from touching the walls of the vessel when they fall. The rims of the cups should not be covered with a thick layer of wax, as if this touches the fuse wire the heat may soften it and cause it to stick. The current for melting the fuse wire is obtained from an 8 V. accumulator, which is connected either directly or if preferred through an ammeter. A fairly large current flows for about $\frac{1}{2}$ sec., when the wire parts and the cup empties. The accumulator should be of the ordinary plate type, not the block type. Provided due attention is paid to the points mentioned above, the arrangement has proved thoroughly reliable.

The salt bridge, the stem of the electrode and the sloping sides of the vessel may if desired be coated with paraffin wax, in order to make sure that the whole of the contents of the cups reaches the main solution and does not adhere to the walls in the form of drops. This is not always necessary, as it is found that as long as the glass is clean all drops flow easily down into the solution. In any case a high-melting wax should be used. We found that a sample melting at 68°

was satisfactory, as water ran freely off the coating without the slightest tendency to adhere, but with all the samples we examined which melted at lower temperatures there was a tendency to leave droplets of water on the surface.

The standard half-cell chosen was the quinhydrone electrode at *p*H 2·0. This is exceedingly constant and reproducible and is very quickly made up. It was of course freshly made for each experiment. A few ml. of a HCl : KCl buffer mixture *p*H 2·0, prepared according to the directions of Clark [1928], are placed in *H* and stirred up with excess of solid quinhydrone.

The whole experimental procedure is as follows. The cups containing the reagents to be added are prepared and suspended as described above, the arrangement being mounted in a stand. The solutions are added to the half-cell as just described, a salt bridge is placed in position and the half-cell closed with its stopper. A glass bead is introduced into the side-bulb *S*, followed by a solution of pyrogallol in water, for which a special curved pipette is used. The enzyme solution to be tested is then placed in the main vessel. Using a curved pipette, strong alkali is then run under the pyrogallol solution in the side-bulb. The main stopper is then inserted and the tube *T* connected with a water pump through a T-tap connected also with a N_2 reservoir. The apparatus is then exhausted, water being run round the water seals at the same time. By turning the T-tap the vessel is twice filled with pure N_2 and exhausted, and after the final exhaustion the tap *T* is closed and detached from the evacuating apparatus. By gently rocking the vessel the bead in *S* is caused to mix the pyrogallol with the alkali. The vessel is then immersed in a water bath at 20° (or room temperature). The method is not suitable for work at 37°, owing to the boiling of the liquids at that temperature in the vacuum. For this reason it is advisable to cool all the solutions under the tap before filling the apparatus. The vessel is held in a retort clamp. If a piece of rubber tube is slipped over the stem of the clamp where it is held in the boss the mounting will be sufficiently elastic to allow it to be shaken gently so as to mix the solution while still holding it securely. The vessel is immersed so deeply in the bath that the water covers the stoppers and only the tubes project; thus there is no possibility of air leaking in. The leads to the potentiometer are then connected with the electrodes and the leads for the accumulator are connected to the correct wires in preparation for the addition of the first reagent. Meanwhile the pyrogallol is absorbing any traces of O_2 which may remain in the vessel, and a short period is allowed for this process to become complete, after which the accumulator can be connected, so as to add the first reagent, and readings begun. The other reagents can then be added at suitable time intervals as desired.

The salt bridges were prepared in lots of fifty at a time as follows. The glass tubes were chemically cleaned, dried and put in an air oven to heat. 100 ml. of saturated KCl solution were heated to boiling and chopped agar added gradually while boiling until no more would dissolve. The solution was then filtered while hot through a hot water funnel, and placed in the hot air oven. The bridges were filled with the hot agar by suction. For this purpose a short length of rubber tube was attached to one end of the bridge and dipped into the agar. A longer rubber tube was attached to the other end for aspiration. The agar was drawn to about $\frac{1}{2}$ in. beyond the end of the glass tube, and it was then allowed to cool in the air. After a few moments it became solidified and the aspirating tube was then cut off about $\frac{1}{2}$ in. beyond the end of the bridge. After all the bridges had been filled in this way they were allowed to cool and then, held by the rubber end pieces, they were dipped momentarily into the hot paraffin wax. They were then allowed to cool for several hours, after which the rubber tips were carefully

split longitudinally with a razor blade and removed, and the projecting agar cut off so as to leave about 1 mm. beyond the ends of the tubes. The purpose of this was to prevent bubbles from forming in the ends of the tube under the vacuum during the experiment, so breaking the contact. The bridges are stored with their ends upwards in a vessel filled with saturated KCl solution. Before use the bridge selected should be rinsed outside with distilled water to remove excess KCl.

The arrangement adopted in this cell avoids any pressure difference tending to drive the agar through the bridge. The agar does not have to withstand the pull of the vacuum, as in certain other types of cells in which one end of the bridge is exposed to atmospheric pressure while the other end is exposed to the vacuum. A source of leakage is thus removed; and at the same time it is possible to use a larger bore for the tubes, with consequently a lower resistance. With bridges filled as described above there is no tendency for the agar to part in the middle of the bridge owing to the formation of gas bubbles under the vacuum.

After use the bridges are emptied by warming slightly and blowing out the agar. They are then cleaned and refilled with fresh agar as already described.

Summary

An anaerobic electrode vessel for oxidation-reduction potential measurements is described. It contains provision for the addition of several reagents successively without opening the apparatus and for removing any traces of O_2 which may be present. It is free from the risk of contamination of the solution with substances from the preceding experiments, which is usually associated with built-in salt bridges.

REFERENCES

Borsook & Schott (1931). *J. biol. Chem.* **92**, 535.
Clark (1928). The Determination of Hydrogen Ions. Baltimore.
Dixon & Zerfas (1940). *Biochem. J.* **34**, 371.
Lehmann (1930). *Skand. Arch. Physiol.* **58**, 173.
Tsukano (1932). *J. Biochem., Tokyo*, **15**, 487.
Wurmser & Geloso (1931). *J. Chim. phys.* **28**, 260.

51. THE ROLE OF COENZYMES IN DEHYDROGENASE SYSTEMS

By MALCOLM DIXON and LEON GROTIUS ZERFAS

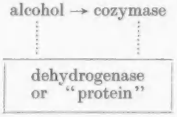
From the Biochemical Laboratory, Cambridge

(*Received 19 January 1940*)

I. Introduction

Many dehydrogenases depend for their action on "coenzymes" (either cozymase or coenzyme II). Owing mainly to the fundamental work of Warburg the mode of action of these substances is now fairly clear. They act by becoming alternately reduced and oxidized and their function in the system is essentially that of a reversible hydrogen acceptor.

Taking the alcohol dehydrogenase system as a typical example, we may picture the process as follows. The substrate (alcohol) forms a highly dissociated compound with a very specific group in the dehydrogenase (or "protein" according to Warburg's terminology). The alcohol when combined is "activated", i.e. made more readily oxidizable. Cozymase also combines with the enzyme, likewise forming a highly dissociated compound. Two H atoms then pass from the combined alcohol to the combined cozymase, as shown by the arrow in the diagram, this being the essential reaction. The oxidized substrate and the



reduced cozymase then leave the enzyme, the former usually to be further metabolized, and the latter to combine either with a flavoprotein or with some other reversible dehydrogenase, where it is reoxidized. The enzyme then combines with further molecules of alcohol and cozymase and the process is repeated. The dehydrogenase thus essentially catalyses a bimolecular reaction between the alcohol and the cozymase:

$$\text{alcohol} + \text{Co} \rightleftharpoons \text{aldehyde} + \text{CoH}_2.$$

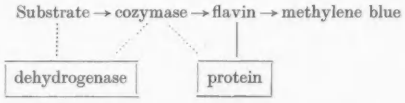
The reaction is reversible, so that presumably the group in the dehydrogenase which combines with the alcohol can also combine with aldehyde, and the group which combines with cozymase can also combine with reduced cozymase.[1]

There is a difference of opinion as to the interpretation of these facts, and two opposing schools of thought have developed during the past two or three

[1] Negelein & Wulff [1937] have measured the affinities of this dehydrogenase for the four substances involved. They find the Michaelis constants (i.e. the concentration of each substance independently at which the dehydrogenase is half saturated with it) to be as follows: cozymase, 0·0001 *M*; reduced cozymase, 0·00003 *M*; aldehyde, 0·0001 *M*; alcohol, 0·024 *M*. Thus the forces uniting the coenzyme with the dehydrogenase are not very different in magnitude from those uniting the aldehyde with the dehydrogenase. A rough calculation shows that under the usual experimental conditions practically the whole of the cozymase is in the free state: at any instant probably less than 1 part in 100 is in combination with the dehydrogenase.

years. The first view, which is held by many workers on enzymes, is that this type of reaction does not differ in any fundamental respect from other bimolecular reactions catalysed by enzymes. The relation of the dehydrogenase protein to cozymase, no less than its relation to alcohol, is essentially that of an enzyme to its substrate. As far as this system is concerned, the cozymase is simply a very efficient H-acceptor. The second view, which is that of the Warburg school, differs fundamentally from this. According to this view the relation of dehydrogenase to cozymase is simply that of protein to prosthetic group: the complex ["protein"+cozymase] is considered to be the enzyme, it is termed a pyridine-protein (since cozymase contains a pyridine ring) and is regarded as a conjugated protein analogous to nucleoproteins, flavoproteins, haemoproteins etc. The cozymase is regarded, not as a substrate or as a coenzyme in the strict sense, but as actually a part of the enzyme itself, and in fact as its active group. The attractiveness of this view lies in the possibility it offers of arriving at a unified conception of enzymes, or at any rate of oxidizing enzymes, as conjugated proteins consisting of protein+prosthetic group. For it brings many of the dehydrogenases into line with such enzymes as peroxidase and catalase, and more particularly with diaphorase and amino-acid oxidase, which have recently been shown to be flavoproteins having a dinucleotide as prosthetic group.

It occurred to us that it might be possible to obtain evidence in favour of one or other of these opposing views by studying the behaviour of these dehydrogenases towards a wider range of H-acceptors than hitherto. Up to the present almost all the work has been done with either flavoprotein or dyes like methylene blue as H-acceptor. In any case, the dyes do not react either with the activated substrate or with the reduced cozymase but only with the reduced flavoprotein, so that their reduction occurs through flavoprotein and, as far as the dehydrogenase is concerned, using a dye is really the same as using flavoprotein itself. Flavoprotein is not reduced by the activated substrate but only by the reduced cozymase, so that the presence of cozymase is essential for the reduction of any of the above H-acceptors to take place:



On the first view—that the cozymase acts in the dehydrogenase system simply as a H-acceptor—it seemed not unlikely that other H-acceptors might be found which, like cozymase, could be directly reduced by the activated substrate; that is to say acceptors for the reduction of which cozymase would not be necessary. In fact there should be two classes of H-acceptors, namely those which like flavoprotein are only reduced in the presence of cozymase and those which are reduced even in its absence. After all, if the activated substrate can reduce cozymase why should it not reduce other substances?

On the other hand, the second view—that the cozymase is the active prosthetic group actually forming part of the enzyme itself—would lead one to expect that no matter what H-acceptors were tested, they should under no circumstances be reduced by the system in the absence of cozymase. The "protein" without cozymase should be catalytically inactive, since the essential part of the enzyme is lacking, just as for example the proteins of catalase and peroxidase are inactive without their haematin prosthetic group.

We therefore decided to take two dehydrogenases which were known to be typical members of the class reacting through cozymase and of which coenzyme-free preparations could be made, and to test their ability to reduce a number of different H-acceptors with and without cozymase. The two which were chosen were the alcohol and malic dehydrogenases of yeast. The former is the enzyme referred to above; the latter catalyses the reaction:

$$\text{malate} + \text{cozymase} = \text{oxaloacetate} + \text{reduced cozymase}.$$

One of us [Dixon, 1926] had already carried out work on a series of substances of very diverse nature which were found to act as H-acceptors for the xanthine dehydrogenase system, and the most important of these were therefore tested with the two selected dehydrogenases. Among these substances were the following: dyes, quinone, alloxan, I_2, H_2O_2, aromatic nitro compounds.

It may be said at once that the results obtained strongly supported the first of the two views outlined above. It was found that with no less than three of these H-acceptors the presence of cozymase was quite unnecessary for the reaction to take place, although with the same enzyme preparations cozymase was absolutely indispensable when methylene blue was the acceptor. In other words, the alleged prosthetic group is only necessary when certain acceptors are used; if the H-acceptor is suitably chosen the "protein" alone can catalyse the reaction without its "prosthetic group". Both the dehydrogenases tested behave in this way.

II. Materials

The greater part of the work was carried out with enzyme preparations made as follows. Delft yeast was plasmolysed with NaCl and disintegrated by grinding for 15 min. in a high-speed ball mill, as described by Ogston & Green [1935]. The resulting viscous suspension was mixed with 3 vol. water and allowed to stand for a short time, after which the insoluble material was centrifuged off and discarded. The soluble enzymes were then purified by fractionation with ammonium sulphate, and the final precipitate was dried *in vacuo* over H_2SO_4 and KOH. This stable preparation contains both the malic and alcohol dehydrogenases. It also contains flavoprotein in sufficient quantities to enable the dehydrogenase systems to reduce methylene blue rapidly after the addition of cozymase. On the other hand, it is entirely coenzyme-free, as shown by the fact that no trace of reduction of methylene blue could be detected in 48 hr. in the absence of added cozymase, either by the preparation alone or after the addition of alcohol or malate. With the addition of alcohol and a small amount of cozymase complete reduction was obtained in under 1 min. The preparation was quite free from reducing material; moreover, no reduction was observed on adding cozymase without alcohol and malate.

When required for use, a portion of the dry preparation was dissolved in $M/15$ phosphate buffer, pH = 7·4, and the insoluble material was centrifuged off and discarded. Except where otherwise stated, 1 ml. of the solution contained the soluble material from 50 mg. of the dry preparation.

The alcohol dehydrogenase has been very highly purified by Negelein & Wulff [1937], and the most important experiments were repeated with an exceedingly pure preparation obtained by their procedure with slight modifications.

The cozymase preparation (purity about 60 %) was made by the method of Green *et al.* [1937].

The only reagent which requires special mention is alloxan. Freshly-made samples were always used, in view of the fact that the substance is somewhat

unstable and undergoes a gradual decomposition in the solid state in the course of a few months, giving a mixture of products. Alloxan was prepared from uric acid by Schlieper's [1845] method, and purified by several recrystallizations from water. Identical results were obtained with a preparation obtained from Hofmann-La Roche. Freshly made up solutions were always used.

III. Potentiometric experiments

Methylene blue, quinone and alloxan have an important property in common, namely they all give well-defined oxidation-reduction potentials at a platinum electrode. That is to say the potential of such an electrode immersed in a solution of any one of these systems is determined (under anaerobic conditions and at a given temperature and *p*H) by the ratio of the concentrations of the reduced and oxidized forms of the substance in question. The most general and most convenient technique for following quantitatively the reduction of any of these H-acceptors is therefore the potentiometric method; any reduction of the acceptor by the enzyme system is clearly shown by a change of the potential in a negative direction. This method was in fact used by Dixon [1926] in studying the reduction of quinone and alloxan by xanthine dehydrogenase.

For this work a special cell had to be devised, since none of those hitherto used was suitable. The main special requirements were: (*a*) a convenient arrangement permitting the successive addition of several reagents (e.g. substrate, H-acceptor, coenzyme etc.) to the solution without introducing any O_2 into the apparatus, (*b*) the complete elimination of every possibility of contamination (especially with traces of cozymase) from a previous experiment, e.g. by diffusion from a salt bridge forming part of the apparatus. The latter point was important, because the possibility of the presence of traces of cozymase would have destroyed the evidential value of our experiments. The apparatus which was devised for this work has been described in the preceding paper [Zerfas & Dixon, 1940]. The details of the method are described there, and it is only necessary here to give the general procedure, which was as follows. The enzyme solution, suitably buffered to *p*H 7·4, was placed in the electrode vessel and the reagents to be added were placed in the small suspended cups. After evacuating and washing out the apparatus several times with pure N_2, it was left evacuated and placed in the water bath at room temperature. After allowing a short time for the pyrogallol in the side-bulb to absorb any residual traces of O_2 the H-acceptor (methylene blue, quinone or alloxan) was mixed with the enzyme solution. The steady potential of the acceptor was rapidly set up, and after waiting for a sufficient period to make sure that no spontaneous reduction of the acceptor was occurring, the reagents could be added at suitable time-intervals, and the course of the reaction followed by the potential measurements. The solution was kept mixed by gentle oscillation during the experiment.

The amount of the acceptor reduced at any instant could theoretically be approximately calculated from the potential reading, making use of the values given in the literature for the normal potentials of the systems. The exact calculation however presents certain difficulties in practice. Fortunately it is not necessary for our present purpose, since the absolute amount of the reduction is unimportant and we only require to know the general course of the reaction. We have therefore preferred simply to take the potential as an index of the extent of the reduction on an arbitrary scale and to plot the observed potentials directly. In the figures therefore the ordinates represent the observed potentials with reference to the standard quinhydrone half-cell (*p*H = 2·0), expressed in mV.,

and the abscissae represent time in min. after adding the acceptor. It will be noticed that a rise in the curve corresponds with a more negative potential and therefore with a reduction of the acceptor.

(a) The alcohol dehydrogenase

Fig. 1 shows the reduction of methylene blue by the alcohol dehydrogenase system. The enzyme and methylene blue were present initially and the potential is that of the methylene blue. On adding the alcohol no change whatever was produced, showing that the system was quite free from cozymase. On the subsequent addition of cozymase the methylene blue was rapidly reduced, as shown by the abrupt rise in the curve. As stated above, this enzyme preparation contained some flavoprotein. The addition of flavoprotein was therefore unnecessary and the methylene blue was reduced immediately upon adding the cozymase.

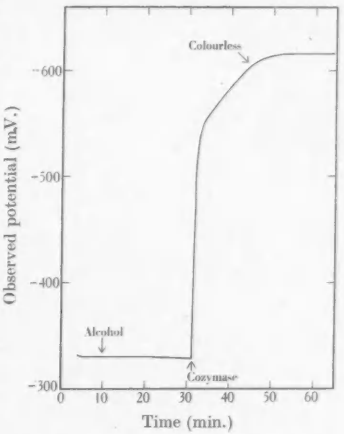


Fig. 1. Reduction of methylene blue by alcohol dehydrogenase. Initially present: 1 ml. enzyme solution + 3 ml. *M*/15 phosphate *p*H 7·3 + (added at zero time) 1 ml. *M*/2000 methylene blue. Added later as shown: 1 ml. *M* ethyl alcohol; 0·2 ml. (= 0·5 mg.) cozymase. Temperature 18°.

Fig. 2 shows a similar experiment carried out with the highly purified enzyme preparation mentioned later, which was free from flavoprotein. In contrast to Fig. 1, no reduction occurred on the addition of cozymase and the presence of flavoprotein as well was necessary. Thus methylene blue is only reduced by the alcohol dehydrogenase if alcohol, cozymase and flavoprotein are all present. This fact is of course very well known already, but it is shown very clearly by this method.

The case of quinone as H-acceptor is slightly different. Fig. 3 shows the reduction of quinone by the alcohol dehydrogenase system. No methylene blue was present. Enzyme and quinhydrone were present initially; the potential is that of the latter and any change shows a reduction of the quinone. As before, no reduction occurred without cozymase. The presence of flavoprotein however was not found to be necessary, so that it appears that the reduced coenzyme can react directly with quinone. The same thing was recently found by Kubowitz [1937] for *o*-quinone, the oxidation product of catechol. Another acceptor of this type is ferricyanide, the reduction of which by dehydrogenases of this kind requires coenzyme but not flavoprotein, as shown by Quastel & Wheatley [1938].

Dickens & McIlwain [1938] also showed that phenazines, unlike other dyes, react directly with reduced coenzyme II and do not require flavoprotein for their reduction.

It should be mentioned that we found quinone to be somewhat toxic to the enzyme if added as such, owing apparently to a direct oxidizing action on the enzyme itself, an effect which has been observed with other enzymes. We found however that if added in the form of quinhydrone it was very much less toxic and this was done in the experiment of Fig. 3.

We may mention here in parenthesis that we found *m*-dinitrobenzene to be reduced by the alcohol dehydrogenase system with the addition of cozymase.

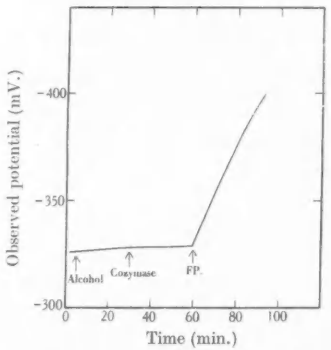


Fig. 2.

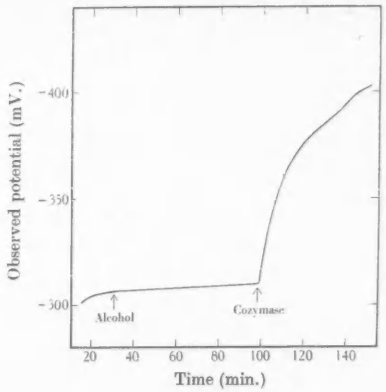


Fig. 3.

Fig. 2. Reduction of methylene blue by highly purified alcohol dehydrogenase. Initially present: 1 ml. purified enzyme solution + 2 ml. *M*/15 phosphate *p*H 7·4 + (added at zero time) 0·5 ml. *M*/2000 methylene blue. Added later as shown: 1 ml. *M* alcohol; 0·2 ml. (= 0·6 mg.) cozymase; 0·1 ml. heart flavoprotein (= 0·2 μg. flavin). Temperature 17°.

Fig. 3. Reduction of quinone by alcohol dehydrogenase. Initially present: 1 ml. enzyme solution + 2 ml. phosphate *p*H 7·3 + (added at zero time) 1 ml. *M*/2000 quinhydrone. Added later as shown: 1 ml. *M* alcohol; 0·2 ml. (= 0·5 mg.) cozymase. Temperature 18°.

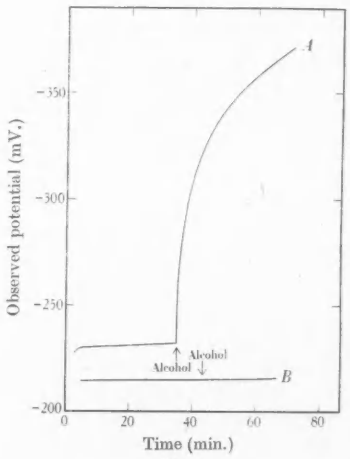


Fig. 4.

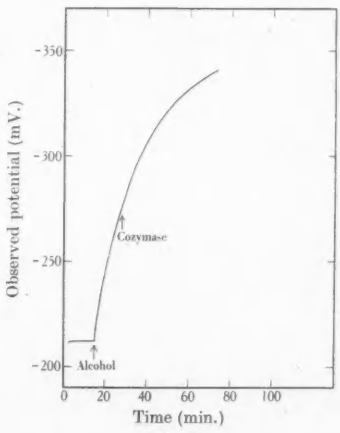


Fig. 5.

Fig. 4. Reduction of alloxan by alcohol dehydrogenase. Curve *A*: initially present: 1 ml. enzyme solution + 3 ml. *M*/15 phosphate *p*H 7·4 + (added at zero time) 0·5 ml. *M*/2000 alloxan. Added later as shown: 1 ml. *M* alcohol. Curve *B*: initially present: 1 ml. enzyme solution previously boiled 3 min. + 2 ml. phosphate *p*H 7·3 + (added at zero time) 1 ml. *M*/2000 alloxan. Added later as shown: 1 ml. *M* alcohol. Temperature 17°.

Fig. 5. Reduction of alloxan by alcohol dehydrogenase. Initially present: 1 ml. enzyme solution + 2 ml. phosphate *p*H 7·3 + (added at zero time) 1 ml. *M*/2000 alloxan. Added later as shown: 1 ml. *M* alcohol; 0·2 ml. (= 0·5 mg.) cozymase.

Lipschitz [1921] has shown that this substance acts as a H-acceptor with dehydrogenases generally in a very similar way to methylene blue. We found that on incubating the dehydrogenase with alcohol, cozymase and a suspension of dinitrobenzene, a strong red colour, due to the reduction product, was produced by the addition of alkali. In a duplicate control without cozymase no trace of colour was produced. The reduction therefore, like that of methylene blue, takes place through cozymase. It was not definitely determined whether it goes through flavoprotein also, but the close similarity between dinitrobenzene and methylene blue in their behaviour with dehydrogenase systems suggests that this is the case.

All the hydrogen acceptors mentioned hitherto, whether they require flavoprotein or not, depend absolutely on the presence of cozymase for their reduction by the alcohol dehydrogenase system. The case of alloxan is very different. Fig. 4, curve *A*, shows a typical experiment. No methylene blue or quinone was present. The enzyme and alloxan were mixed initially and the steady potential due to the alloxan was quickly set up. In contrast to the other cases, as soon as the alcohol was added a rapid reduction of the alloxan occurred, in spite of the fact that cozymase was completely absent, as shown by the previous results. Thus with this acceptor the "protein" part of the system alone can catalyse the reaction and cozymase is not necessary. This striking result has been obtained with more than a dozen different preparations of the enzyme and with several different samples of alloxan. Moreover, the reaction is not accelerated if cozymase is added while it is proceeding, as Fig. 5 shows.

The reaction is of course due to the action of the enzyme and no effect is obtained if the enzyme solution is first boiled, as shown in Fig. 4, curve *B*. In different preparations the rate of change of potential runs roughly parallel with the alcohol dehydrogenase activity as tested in the usual way by the Thunberg technique. It may also be mentioned that the enzyme solutions alone, in the absence of alloxan or any other added H-acceptor, gave no potential change on the addition of alcohol. On adding both alcohol and cozymase a slight drift of potential in a negative direction was observed, which was probably due to the small amounts of flavoprotein present in the enzyme solutions. This effect was negligible in comparison with those obtained with the added aceptors.

Like quinone, alloxan was found to have a toxic action on the enzyme, an effect which has been previously found with other dehydrogenases. This does not seem to be marked with the alloxan concentrations used in these experiments, but much higher concentrations should not be used if the toxic effect is to be avoided.[1]

In order to establish our contention, we have to show not only that alloxan is reduced by the system, but that cozymase is completely absent. For this purpose direct chemical tests are of course useless, owing to their comparative insensitivity, and the most stringent test of its absence is the non-occurrence of some reaction which is rapidly catalysed by it. The most suitable reaction for this purpose is the reduction of methylene blue by dehydrogenases in presence of flavoprotein. If no reduction of the dye occurs in the absence of added cozymase, whereas the addition of a little cozymase causes a rapid reduction, it can be taken as proved that no cozymase was present initially. Such evidence

[1] The inactivation by quinone and alloxan is presumably due to the oxidation of some group in the enzyme. We have obtained some indications that after partial inactivation of the enzyme by moderately high concentrations of alloxan it may be reactivated by incubation with alcohol and cozymase. It is possible that this is due to a reduction of the group in question by reduced cozymase.

has already been presented in Fig. 1, which shows that there is not the slightest trace of reduction of methylene blue by the enzyme solution containing flavoprotein and alcohol, but a very rapid reduction on adding cozymase. This can be shown even more convincingly by the Thunberg method. In view of the results given in Table 1 there can be no doubt that our preparations were completely free from traces of cozymase. The catalytic action of small amounts of cozymase is not enhanced by the addition of alloxan.

Table 1

Quantities as follows: enzyme 1 ml. (=50 mg. original prep.); methylene blue 0·5 ml. $M/2000$; alcohol 1 ml. M; malate 1 ml. $M/10$; cozymase 0·5 ml. (=0·5 mg.); total volume 5 ml., made up with $M/15$ phosphate buffer pH 7·3. Temperature 37°. Thunberg tubes with hollow stoppers were used and the methylene blue and cozymase were placed in the stoppers. The tubes were alternately evacuated and filled with pure N_2 twice and finally evacuated, so as to remove all traces of O_2. The contents of the tubes were mixed after they had attained the temperature of the thermostat.

Tube 1. Enzyme + methylene blue	No trace of reduction in 48 hr.
Tube 2. Enzyme + methylene blue + alcohol	No trace of reduction in 48 hr.
Tube 3. Enzyme + methylene blue + malate	No trace of reduction in 48 hr.
Tube 4. Enzyme + methylene blue + alcohol + cozymase	Complete reduction in 45 sec.
Tube 5. Enzyme + methylene blue + malate + cozymase	Complete reduction in 6 min.

Although this evidence would seem to be adequate, we have carried it still further by making a very highly purified preparation of alcohol dehydrogenase. Negelein & Wulff [1937], by a complicated series of fractionations and precipitations, have obtained the enzyme from autolysed brewer's bottom yeast in a pure crystalline form, and we have made use of this method. In spite of every effort to follow their procedure exactly, we did not succeed in obtaining crystalline preparations or in attaining the very high activities reported by them, probably owing to the use of a different yeast. The yeast available to us was a bottom yeast obtained from Belgium, and the enzyme was found at almost every stage to precipitate under slightly different conditions from those given by Negelein & Wulff, so that many slight modifications were necessary. Nevertheless, by carrying through the whole procedure with these modifications we obtained a highly purified and very active preparation of alcohol dehydrogenase, completely free, as far as we could determine, from every other enzyme and coenzyme and from flavoprotein.

It is well known that cozymase undergoes rapid destruction when yeast is ground up or autolysed, and even in the early stages of purification the preparations were found to be free from any trace of cozymase by the methylene blue test. Even if minute traces had been present, we consider that it is inconceivable that any could have survived the subsequent thorough-going purification processes.

With this preparation, diluted to give a reasonable activity, the experiment of Fig. 6 was carried out. It will be seen that the purified enzyme "protein", without any additions, is able to catalyse the reaction between alcohol and alloxan just as the ordinary preparations do.

The reduction of alloxan occurring in the first few minutes of the experiment, before any alcohol had been added, was only obtained with this preparation. It is most probably due to traces of alcohol already present in the enzyme preparation. The last step in the purification of the enzyme is a precipitation by

alcohol, and it is possible that the preparation still contained a certain amount. This would cause a slight initial reduction of alloxan, such as that shown by the curve, which however soon comes to a standstill and does not interfere in any way with the main part of the experiment.

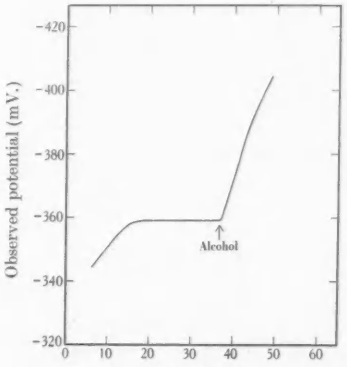


Fig. 6.

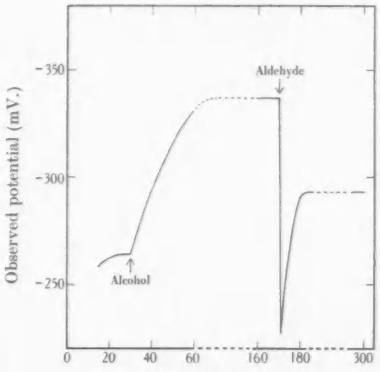


Fig. 7.

Fig. 6. Reduction of alloxan by highly purified alcohol dehydrogenase. Initially present: 1 ml. purified enzyme solution + 2 ml. *M*/15 phosphate *p*H 7·4 + (added at zero time) 0·1 ml. *M*/50 alloxan. Added later as shown: 0·5 ml. *M* alcohol. Temperature 19°.

Fig. 7. Reversibility of reaction between alcohol dehydrogenase and alloxan. Initially present: 1 ml. enzyme solution + 2 ml. phosphate *p*H 7·3 + (added at zero time) 1 ml. *M*/2000 alloxan. Added later as shown: 1 ml. *M*/10 alcohol; 1 ml. *M*/5 acetaldehyde.

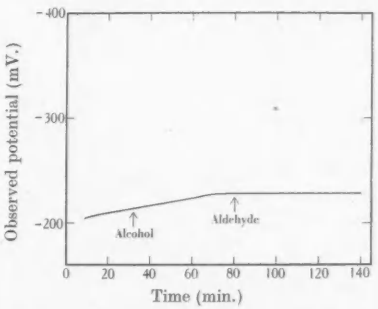


Fig. 8. Repetition with boiled enzyme. Details exactly as for Fig. 7, except that the enzyme solution was previously boiled 3 min.

Before proceeding to the malic dehydrogenase a further observation on the alcohol dehydrogenase may be of interest. The reaction between alcohol and cozymase catalysed by the enzyme is reversible and it was thought that this might also be the case for the reaction between alcohol and alloxan:

$$\text{alcohol} + \text{alloxan} = \text{aldehyde} + \text{dialuric acid}.$$

The experiment shown in Fig. 7 seems to show that this is so, although we do not wish to lay too much stress on the point, as this was the only such experiment carried out. After allowing the alcohol (one-tenth usual amount) and alloxan to react, neutral acetaldehyde was tipped in and, after a transient "kick", produced a definite change of potential in the expected direction. This effect was definitely enzymic, as Fig. 8 shows that it is not observed with boiled enzyme solution.

(b) The malic dehydrogenase

The behaviour of the malic dehydrogenase is similar in all essential respects to that of the alcohol system. Figs. 9–11 show the reductions of methylene blue,

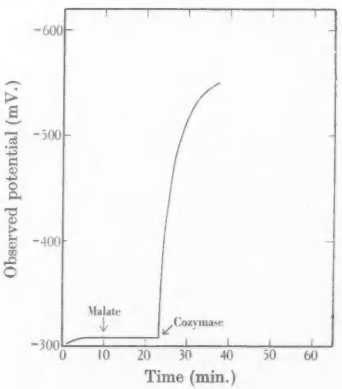


Fig. 9.

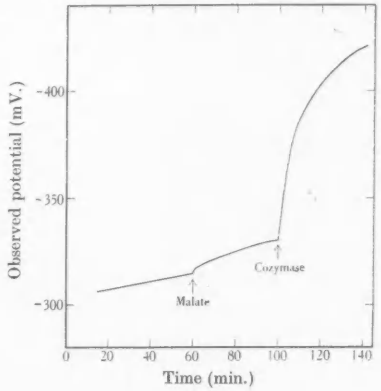


Fig. 10.

Fig. 9. Reduction of methylene blue by malic dehydrogenase. Initially present: 1 ml. enzyme solution + 2 ml. phosphate *p*H 7·3 + (added at zero time) 1 ml. *M*/2000 methylene blue. Added later as shown: 1 ml. *M*/10 malate; 0·2 ml. (=0·5 mg.) cozymase.

Fig. 10. Reduction of quinone by malic dehydrogenase. Initially present: 1 ml. enzyme solution + 3 ml. *M*/15 phosphate *p*H 7·4 + (added at zero time) 1 ml. *M*/2000 quinhydrone. Added later as shown: 0·1 ml. *M* malate; 0·2 ml. (=0·5 mg.) cozymase.

quinone and alloxan respectively, and may be compared with Figs. 1, 3 and 4. No purification of the malic enzyme comparable with that of the alcohol enzyme has yet been accomplished, so that we have no experiment corresponding with Fig. 2.

It will be seen that, as in the case of the alcohol dehydrogenase, cozymase is essential when methylene blue or quinone are used as H-acceptors; but when alloxan is used as acceptor the oxidation of malate is catalysed by the dehydrogenase in the complete absence of cozymase. The absence of cozymase from this enzyme preparation has already been shown by the results of Table 1. The reduction of alloxan is a little slower than it is with the alcohol dehydrogenase, as the malic enzyme is less active (cf. Table 1). It is easy to show that the oxidation of malate by alloxan, like that of alcohol, is enzymic, for no effect is obtained with a boiled enzyme solution, as shown in Fig. 11, curve *B*. There is no doubt that the catalysis of the reaction between malate and alloxan is due to the malic dehydrogenase; a preparation of alcohol dehydrogenase which has been freed from malic dehydrogenase gives no reduction of alloxan by malate, but a rapid reduction by alcohol.

Thus we have shown that two typical members of the group of cozymase-dependent dehydrogenases (or "pyridine-proteins") are active without cozymase, provided that the H-acceptor is suitably chosen.

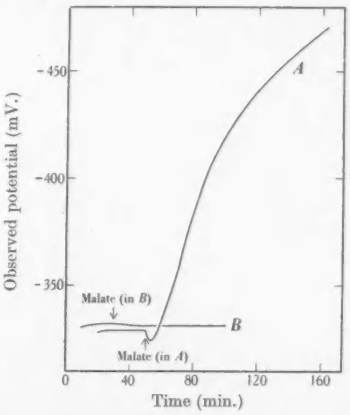


Fig. 11. Reduction of alloxan by malic dehydrogenase. Initially present: 1 ml. enzyme solution + 2 ml. phosphate *p*H 7·3 + (added at zero time) 1 ml. *M*/2000 alloxan. Added later as shown: 1 ml. *M*/10 malate. Curve *A* with fresh enzyme; curve *B* with boiled enzyme. Temperature 18°.

(*c*) *Other dehydrogenases*

Similar results were obtained with a number of other dehydrogenases. The xanthine [Dixon, 1926], succinic and yeast lactic dehydrogenases were all found

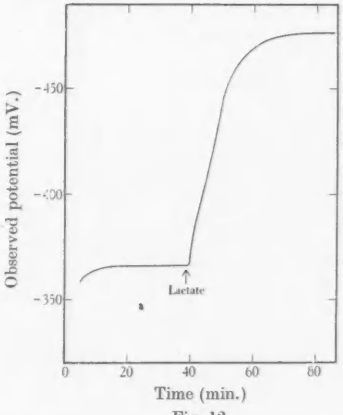


Fig. 12.

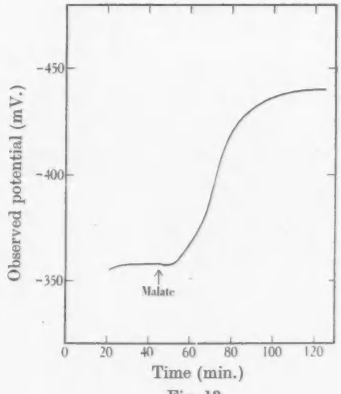


Fig. 13.

Fig. 12. Reduction of alloxan by lactic dehydrogenase of heart. Initially present: 1 ml. pig heart enzyme + 2 ml. *M*/15 phosphate *p*H 7·4 + (added at zero time) 0·3 ml. *M*/142 alloxan. Added later as shown: 0·5 ml. *M* lactate. Temperature 20°.

Fig. 13. Reduction of alloxan by malic dehydrogenase of heart. Details as for Fig. 12, but with 0·5 ml. *M* malate instead of the lactate.

to reduce alloxan in the absence of cozymase, but this is not surprising, as they do not depend upon cozymase even for their reduction of methylene blue. The lactic and malic dehydrogenases of heart muscle, however, are known to reduce dyes only in presence of cozymase. With alloxan, nevertheless, cozymase is unnecessary, just as with the two dehydrogenases first dealt with, as shown in Figs. 12 and 13 respectively. These experiments were carried out on a preparation of the enzyme obtained from pig heart muscle which was kindly given to us by Dr Dewan. These experiments have not the same evidential value as those given above, as it was not possible to be equally certain that all traces of cozymase were absent. Nevertheless, the results obtained by the Thunberg method with this preparation, given in Table 2, show that such traces, if present, are very small, and it is highly probable that these dehydrogenases also can dispense with cozymase under such conditions. The slight residual reduction is probably not due to cozymase but to traces of spontaneously reducing substances.

Table 2

Quantities as follows: enzyme 1 ml.; methylene blue 0·5 ml. $M/2000$; Na malate 0·5 ml. M; Na lactate 0·5 ml. M; cozymase 0·2 ml. (=0·6 mg.); total volume 5 ml., made up with $M/15$ phosphate buffer pH 7·4. Temperature 37°. Procedure as in Table 1.

		Reduction time
Tube 1.	Enzyme + methylene blue + malate + cozymase	70 sec.
Tube 2.	Enzyme + methylene blue + lactate + cozymase	100 sec.
Tube 3.	Enzyme + methylene blue + malate	3 hr.
Tube 4.	Enzyme + methylene blue + cozymase	4 hr.

IV. Estimations of the oxidation product

Not wishing to rely entirely on potentiometric measurements, we have confirmed the above results and extended them to some other H-acceptors by an entirely independent method. In the case of the malic dehydrogenase, the reaction

$$\text{malate} + \text{alloxan} = \text{oxaloacetate} + \text{dialuric acid}$$

can obviously be followed either by measuring the degree of reduction of the alloxan, as above, or by estimating the amount of oxaloacetate formed. Small amounts of oxaloacetate are readily estimated manometrically by the aniline citrate method worked out by Ostern [1933] and Edson [1935], and we have made use of this method in order to study the behaviour of the malic dehydrogenase towards not only the acceptors already dealt with but also others for which the potentiometric method is not suitable.

It was found that by making several slight modifications in the method its reliability and sensitivity could be considerably increased. One of the chief causes of inaccuracies is the pressure change, not completely explained, which develops for some minutes after the aniline-citrate mixture is diluted by mixing with the main solution, even in the absence of oxaloacetate. This is usually regarded as a "blank" to be subtracted from the reading with oxaloacetate. The compensation is not always perfect, however, as it is difficult to duplicate the conditions exactly in the control manometer. We have carried out most of our estimations as follows. Barcroft differential manometers were used, and the aniline citrate was added to both flasks of each manometer. 2 ml. water + 0·3 ml. glacial acetic acid were placed in the main body of the flasks, the aniline citrate (0·3 ml. of a mixture of equal parts of aniline and 50 % citric acid) was mixed with this at the beginning and the manometers shaken in the thermostat at 37°

for 10 min., with the taps open, by which time equilibrium had been reached. The taps were then closed, and the initial readings taken. Only then was the solution to be tested, acidified with glacial acetic acid, tipped into the solution in the right-hand flask, an equal amount of water containing acetic acid being added to the left-hand flask. The reaction was found to be complete after a further 20 min. shaking and the final readings were then taken. The manometers were read to the nearest 0·1 mm.

The use of a temperature of 37° reduced the time required for the estimations, but it is known that oxaloacetic acid decomposes spontaneously to some extent at this temperature, and experiments were therefore carried out with a solution containing a known amount of oxaloacetate in order to see whether this source of error was serious. As can be seen from Table 3, the magnitude of the error was unimportant for our purpose.[1] The results are expressed in μl. of CO_2, 1 μmol. oxaloacetate corresponding to 22·4 μl. CO_2. The table also shows that very small amounts of oxaloacetate added to the enzyme preparation can be determined satisfactorily. The blank for the enzyme was found to be zero. Some samples of malate however were found to give a small consistent blank of a few μl., even in absence of the enzyme, and the figures given below have been corrected for this where necessary.

Table 3

	Oxaloacetate added μl.	Oxaloacetate found μl.
Oxaloacetate	170	160
Enzyme + oxaloacetate	17	16·5
Enzyme + oxaloacetate	17	14
Enzyme	0	0

In carrying out the main experiments the dehydrogenase, malate and H-acceptor were incubated together at 30° under anaerobic conditions to allow the reaction to take place. The enzyme was then destroyed by adding glacial acetic acid and the oxaloacetic acid formed was estimated in samples of the liquid by the method just described. The malic dehydrogenase preparation used in these experiments was the same as that used for the potentiometric work.

The results obtained with alloxan as acceptor are given in Table 4. No cozymase was present in any of these experiments. Where two figures are given they refer to separate experiments. A + sign denotes that the substance in question was present in the concentration specified, a − sign indicates that it was absent.

Table 4

Final volume 25 ml. in each case, made up with $M/15$ phosphate buffer pH 7·4, and containing 500 mg. enzyme preparation and the following concentrations of the other reagents, where present: alloxan, 0·001 M; Na malate, 0·02 M except in the controls without alloxan, where it was increased to 0·2 M.

Enzyme	Malate	Alloxan	Time of incubation min.	Oxaloacetate found μl.
+	+	+	0	0; 5
+	+	+	10	40
+	+	+	20	92
+	+	+	60	93; 94
+	−	+	60	0
+	+	−	60	0; 0
Boiled	+	+	60	0

[1] The results agree with those of Breusch [1939] just published.

These results give a complete confirmation of those obtained by the potentiometric method. No oxaloacetate is formed during the incubation when either the malate or the alloxan is missing, or when both are incubated with boiled enzyme; none is found when all the components are present but the mixture is given no time to react. But when the complete system is incubated oxaloacetate is fairly rapidly produced. Thus there is no doubt that the malic dehydrogenase can catalyse the oxidation of malate to oxaloacetate by alloxan in the absence of cozymase.

The results with alloxan can be contrasted with those obtained with methylene blue as acceptor, shown in the first part of Table 5. The fact that oxaloacetate

Table 5

Conditions as in Table 4, except that the concentration of malate in the mixture was 0·04 *M*. The same enzyme preparation was used. (diBrPhI = 2:6-dibromophenolindophenol.)

Enzyme	Malate	Cozymase	Acceptor	Conc. of acceptor	Incubation time min.	Oxaloacetate found μl.
+	+	–	Meth. blue	0·0005 *M*	60	0
+	+	3 mg.	Meth. blue	0·0005 *M*	20	147
+	+	4·7 mg.	Meth. blue	0·0005 *M*	20	169
+	+	–	Quinhydrone	0·0005 *M*	20	0; 5
+	–	–	Quinhydrone	0·0005 *M*	20	0
+	+	–	Iodine	0·0005 *M*	20	114; 94
+	+	–	Iodine	0·00065 *M*	20	138
–	+	–	Iodine	0·00065 *M*	20	−4
+	+	–	–	0	20	0
+	+	–	H_2O_2	0·002 *M*	20	97
+	+	–	–	0	20	5
+	+	–	$K_3Fe(CN)_6$	0·0005 *M*	20	0
+	+	–	$KMnO_4$	0·0005 *M*	20	0
+	+	–	diBrPhI	0·001 *M*	20	69
+	–	–	diBrPhI	0·001 *M*	20	0

was rapidly formed in presence of cozymase and methylene blue, but that none at all is formed with methylene blue alone, even with an incubation three times as long, gives further evidence of the freedom of the enzyme preparation from cozymase. Some figures are also given for quinhydrone as acceptor, but it is possible that these are not so reliable as the others, as quinone seems to interfere to some extent with the aniline citrate reaction.

Certain other substances which had previously been found to act as H-acceptors with xanthine dehydrogenase are also included in the table. These substances are all reduced rather rapidly even in the absence of malate by substances present in the enzyme preparation. This made it impossible to study their reaction with the malate system by the usual method, i.e. by measuring their rate of reduction. They could however be studied by estimating the oxaloacetate formed, which would show whether they reacted with the malic system even if they were mainly used up in oxidizing other substances, or in the case of H_2O_2 by catalase, which was present in small amounts. In actual fact no less than three of these substances (iodine, H_2O_2 and dibromophenolindophenol) behave like alloxan, in that the malic dehydrogenase does not need cozymase in order to react with them. Iodine acts particularly well, although it has no action in the absence of the enzyme. The effective concentration was certainly much less than that given, as it was reduced fairly rapidly by other substances present in the enzyme preparation. In higher concentrations it was found to be toxic to the enzyme. H_2O_2 also acts as a direct H-acceptor, as with

xanthine dehydrogenase; it was not found to be toxic, possibly owing to the presence of some catalase. It is probably more effective than it appears, since the catalase no doubt reduced its concentration to a much lower figure. Ferricyanide had already been shown by Quastel & Wheatley to require cozymase in order to react with cozymase-dependent dehydrogenases.

The case of the indophenol dye is interesting, as all the other dyes hitherto tested have been found to react with such systems only through cozymase. This dye has an unusually high oxidation potential, but it is not clear whether its behaviour is due to this or whether other members of the indophenol series would behave similarly. Owing to its high potential it is reduced fairly rapidly by the enzyme solution itself and the presence or absence of malate has little effect on its reduction time as determined by the Thunberg method. Nevertheless, at the conclusion of the Thunberg experiments also the presence of oxaloacetate was definitely confirmed in the tubes containing the enzyme, malate and indophenol, though none could be detected when one of the components was missing. Thus this dye can be reduced by the activated malate directly. But it can also be reduced, like other dyes, through cozymase and flavoprotein, for the addition of cozymase did accelerate its reduction by the malic system.

It should be possible to study the reaction of alloxan with the alcohol dehydrogenase, like the malic dehydrogenase, by estimations of the oxidation product. Owing to certain technical difficulties, however, we have not obtained satisfactory quantitative results with this system. The difficulty is in estimating very small amounts of aldehyde in solutions of the enzyme preparation containing comparatively large amounts of alcohol. Only small amounts of aldehyde can be expected, for the alloxan concentration is small and cannot be increased because of its toxicity to the enzyme. The alcohol concentration, on the other hand, must be kept up owing to the rather small affinity of the enzyme for alcohol. We have not succeeded in finding a satisfactory method for use under these conditions. Though we were able, by the isolation of the dinitrophenylhydrazone from distillates, to obtain qualitative evidence that aldehyde was formed in the reaction with alloxan in the absence of cozymase, we have no quantitative results on this system to offer comparable with Tables 4 and 5. In view of the definiteness of the potentiometric results, however, and the close similarity in the behaviours of the two systems, we do not feel that this is a serious omission.

Further comments on the above experiments

It has been shown that several of the substances tested can replace cozymase as H-acceptors in the dehydrogenase systems. In order to avoid misunderstandings it should be made clear that we do not claim that these substances can act as coenzymes for these systems. Cozymase acts as a coenzyme because it is not only reduced by the dehydrogenases but is then reoxidized by other systems (flavoproteins, etc.). Alloxan and the other substances do not seem to act as coenzymes because, although they are reduced like cozymase by the dehydrogenases, they are not re-oxidized by the other systems. In the case of alloxan it might be thought that it should act as a coenzyme, because its reduced form, dialuric acid, can reduce methylene blue and is also oxidized by O_2. No coenzyme action, however, could be observed. But dialuric acid itself, when added to the enzyme solution, failed to reduce methylene blue or to take up O_2, although it did so readily in the absence of the enzyme. The fact is that the oxidation of dialuric acid is due to a rather complicated heavy-metal catalysis, and it is well known that many proteins and tissue preparations are apt to

inhibit such reactions, presumably by forming inactive complexes with the catalytic metals. There is no doubt that this, and not a failure to become reduced by the dehydrogenase, is the reason why alloxan did not act as a co-enzyme in our experiments. The mechanism of the oxidation of dialuric acid is a quite separate problem, with which we are not at the moment concerned.

By a curious coincidence, while this work was in progress Bernheim [1938] published a paper showing that the aerobic oxidation of alcohol by liver suspensions is strongly catalysed by alloxan. In some cases the rate of oxidation was increased by 1000% by alloxan in a concentration of $M/10{,}000$. This effect was not explained, but it seems probable that it is connected with the effects reported here, though one cannot be dogmatic on the point, for Bernheim obtained negative results with the oxidation of lactate, whereas in our experiments (Fig. 12) the lactic dehydrogenase behaved towards alloxan in the same way as the alcohol dehydrogenase.

Bernheim tested alloxan because of its relation to the flavins; it is used in their synthesis and can be regarded as forming part of the flavin molecule. It has been suggested to us that our results may be connected with that relationship. We do not think that this is the case, for the following reasons: (1) The relationship is not really very close from an oxidation-reduction point of view: the part of the alloxan molecule which is active in these reactions, namely the group which is reduced to form dialuric acid, has disappeared in the flavin molecule. Also the flavin group itself, without the carbohydrate side chain, phosphoric acid and protein, is inactive in any case. (2) The suggested relationship is not to cozymase but to flavin, which does not react with the dehydrogenases and has quite a different function. (3) We have shown that other quite unrelated substances can act in the same way as alloxan.

It should be made clear that the substances which we have shown to act like cozymase as direct H-acceptors do not equal cozymase in efficiency. Data for an exact comparison are lacking, but in the case of the malic system a rough calculation of relative velocities can be made from the data of Tables 1 and 4, making due allowance for the difference in conditions. This indicates that cozymase is reduced from three to six times as rapidly as alloxan by the malic enzyme under similar conditions. The difference is probably not so great in the case of I_2, but the effective concentration of I_2 cannot be determined. It is of course to be expected that the enzyme would react most readily with its natural H-acceptor, cozymase.

Finally, it may be objected that alloxan is unstable in aqueous solution. Richardson & Cannan [1929] have stated, purely on the basis of a drift in their observed potentials and without any chemical evidence, that in alkaline and even in neutral solutions alloxan rapidly changes into alloxanic acid, the half-life period of alloxan at *p*H 7 being only 1 min. at 30°. This is definitely not the case. Hill & Michaelis [1933] have shown that the potential drift in question is abolished by the addition of small amounts of iron salts, which catalyse the reaction of the system with the electrode. It is therefore due to some other cause. Alloxan is in fact reasonably stable in neutral solution. The change to alloxanic acid, which involves the opening of the 6-membered ring and reclosure to give a 5-membered ring, requires heating of the alloxan with strong alkali. Behrend & Zieger [1915] have shown that if KOH is added to a solution of alloxan (*p*H about 8) the product which crystallizes after some time (in 70% yield) is the pure potassium salt of alloxan and not that of alloxanic acid. The potassium salt of alloxanic acid is produced only on allowing the mixture to stand "for some hours" or on warming. Liebig & Wöhler [1841] showed that

on boiling aqueous solutions of alloxan it is decomposed into alloxantin, parabanic acid and CO_2, but the process is a slow one, even at the boiling point, and would certainly be negligible at room temperature. It is true that alloxan solutions undergo a change in conductivity on standing for many hours [Wood, 1906]. The change is most likely a simple one, such as the enolization HN—CO $\rightleftharpoons$ N=C(OH) which occurs in the analogous purine compounds.

The question does not really affect our work in any vital respect, for it is immaterial to our argument whether the substance which acts as a direct H-acceptor with the dehydrogenases is alloxan itself or some other substance derived from it. In particular, the most likely derivative, alloxantin, could have been used in our experiments instead of alloxan, as it bears the same relation to alloxan as quinhydrone does to quinone. We have indeed repeated the most important potentiometric experiments using alloxantin and obtained similar results. We believe, nevertheless, that no appreciable decomposition can have taken place under the conditions of our experiments, which were of short duration, at almost neutral *p*H and at reasonably low temperatures. Moreover, our results do not depend only on experiments with alloxan.

V. Discussion

The view that the two coenzymes form the prosthetic groups of dehydrogenases is undoubtedly very attractive. The close analogy with flavoprotein enzymes like the *d*-amino-acid oxidase, in which the prosthetic flavin group is chemically related to cozymase and is reduced by the substrate in much the same way, is admittedly a strong argument in its favour. It is however the only argument advanced in its favour, and there are several strong arguments against it. Even this argument from analogy can be used in two ways, for nucleotides not only form prosthetic groups but also act as carrier-substrates. For instance, adenylic acid, which also is chemically related to cozymase, plays very much the same role in phosphate transport as cozymase does in hydrogen transport: it comes into relation with many enzymes, but it is not assigned as prosthetic group to any of them. Cozymase likewise does not remain permanently attached to any one enzyme; it comes into relation with many enzymes, but like adenylic acid is for the most part free in solution.

The following are some of the arguments which may be advanced against the view that cozymase is to be regarded as the prosthetic group of the dehydrogenases with which it acts:

(1) The type of union between coenzyme and dehydrogenase is that which we have come to associate with the union of enzyme and substrate, rather than the type which is met with in conjugated proteins. In conjugated proteins the prosthetic group is attached to the protein by a definite chemical combination, and it can only be separated from the protein by splitting with acid or some equally drastic process. The combination of coenzyme with dehydrogenase, like that of substrate with enzyme, is highly dissociated, almost instantaneously reversible when the concentration of free coenzyme is altered and comes to a definite equilibrium when only a small part of the total coenzyme is combined. The value of the affinity of the enzyme for cozymase is quite a common one for enzyme-substrate combinations. Thus the analogy with conjugated proteins breaks down in this respect.

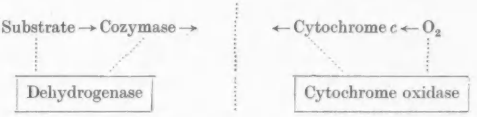
(2) The dehydrogenase catalyses the reaction between two substances, the specific substrate of the enzyme and cozymase (see the introduction), and it is difficult to see why one should be chosen as the prosthetic group of the enzyme

LINDA HALL LIBRARY
KANSAS CITY, MO.

rather than the other. There seems in fact to be no criterion for such a distinction. They both combine dissociably with the enzyme in much the same way; as we have seen the affinity of the enzyme for aldehyde is the same as its affinity for cozymase, so that the strength of the forces involved is the same in each case; the combination is specific in both cases—if anything it is less specific for cozymase, which combines with many different enzymes. If we are to speak of "pyridine-proteins" it seems that we should be equally justified in speaking of "aldehyde-proteins", a term which would include the Schardinger enzyme, the aldehyde oxidases of liver and potato, alcohol dehydrogenase and aldehyde mutase. The fact that cozymase has a carrier function in the cell, which most of the other substrates have not, is not a valid reason for making the distinction; for the fact that the cozymase goes on to react with other systems after leaving the dehydrogenase is irrelevant so far as the dehydrogenase itself is concerned.

(3) There is evidence [Haas, 1937] that cozymase combines with flavoprotein when it reduces the flavin group, just as it combines with the dehydrogenase when it oxidizes the substrate. If it is to be regarded as a prosthetic group when combined with the dehydrogenase it must logically be so regarded when combined with flavoprotein also. Thus the flavoprotein must then be considered a pyridine-protein, and would have two prosthetic groups.

(4) The two terminal systems of the main catalytic chain in the respiratory mechanism can almost be regarded as functionally mirror images of one another:



The relationship of cytochrome *c* to cytochrome oxidase shows a close analogy (when we substitute oxidation for reduction) with that of cozymase to the dehydrogenase. Logically therefore we should have to consider cytochrome *c* as the prosthetic group of cytochrome oxidase.

(5) Cozymase is hydrolysed by a phosphatase, which of course combines with it in order to bring about the reaction. There seems to be no logical basis for making a distinction between the combination of cozymase with phosphatase and its combination with dehydrogenase, so that the former would also have to be considered a pyridine-protein, though only when it catalyses this particular reaction. Thus it would sometimes be a pyridine-protein and sometimes not, just as the dehydrogenase would have to be considered as sometimes a pyridine-protein and sometimes not (e.g. when it catalyses the reaction with alloxan, etc.).

The three last points are given as examples of the confusion which would follow from the logical application of the idea.

(6) In addition to its normal reaction, succinic dehydrogenase catalyses the reverse reaction:

$$\text{fumarate} + \text{CoH}_2 = \text{succinate} + \text{Co},$$

where Co represents cozymase [Dewan & Green, 1937]. This reaction is exactly analogous to the reverse reaction of the alcohol dehydrogenase:

$$\text{aldehyde} + \text{CoH}_2 = \text{alcohol} + \text{Co}$$

and the corresponding reactions of other cozymase-dependent dehydrogenases. The closeness of the analogy is such that if the cozymase is to be regarded as the prosthetic group in these cases it must also be so regarded in the succinic system.

But the succinic dehydrogenase is one of the coenzyme-independent enzymes; it is not a pyridine-protein, for its normal forward reaction succinate + methylene blue (etc.) = fumarate + MBH_2 (etc.) is quite independent of the presence of either of the coenzymes.

(7) The most fundamental distinction between a substrate and a prosthetic group of an enzyme is as follows.[1] If to a solution containing the substance in question (prosthetic group or substrate), together with excess of the specific substrate of the enzyme, a small amount of the enzyme protein is added and allowed time to act, an unlimited amount of the substance will be reduced if it is a substrate, whereas if it forms the prosthetic group the amount reduced cannot be more than stoichiometrically equivalent to the enzyme protein added. That is to say if we add 100 molecules of the enzyme protein, each molecule of which combines with one molecule of the substance as its prosthetic group, not more than 100 molecules of the substance can become reduced. On the other hand if the substance is a substrate of the enzyme, 100 enzyme molecules, given time, could reduce an unlimited amount of it. Let us apply this test, for purposes of comparison, to the amino-acid oxidase and the alcohol dehydrogenase. Warburg & Christian [1938] have shown that if a small amount of the protein of the oxidase is added to a mixture of flavin-adenine-dinucleotide and alanine no visible reduction of the flavin occurs. Only that small fraction which is combined as part of the enzyme is reduced by the alanine: the free flavin-dinucleotide does not form a substrate for the enzyme, and is not reduced. In order to reduce all of the dinucleotide an amount of the oxidase protein equivalent to it must be added, when it all becomes combined as part of the enzyme. If on the other hand a small amount of the alcohol dehydrogenase protein is added to a mixture of cozymase and alcohol the whole of the cozymase is rapidly reduced. By this criterion therefore the flavin nucleotide forms the prosthetic group of the amino-acid oxidase, whereas cozymase is definitely a substrate of the dehydrogenase.

(8) The complex [dehydrogenase + cozymase], *as such*, cannot be regarded as a catalyst in the strict sense. The dehydrogenase is a true catalyst, catalysing the reaction between cozymase and, say, alcohol. The cozymase is also a true catalyst, catalysing the reaction between the dehydrogenase-alcohol complex and flavoprotein. The dehydrogenase-cozymase complex, however, cannot as such be regarded as catalysing any reaction. One part of the complex is reduced by alcohol; the complex then breaks down, the cozymase part migrates to form new complexes with other systems, while the dehydrogenase part combines with another cozymase molecule and the process is repeated. If the complex is not strictly a catalyst it seems undesirable to apply the term enzyme to it.

(9) The final argument is provided by the observations described in this paper. We have shown that the dehydrogenase proteins, under proper conditions, can bring about their respective oxidations in the complete absence of the alleged prosthetic group. They are therefore complete enzymes in themselves. A prosthetic group which does not form an essential part of the enzyme seems to be a contradiction in terms, and since the enzymes can act without cozymase we do not think it is any longer possible to regard cozymase as their prosthetic group. The term pyridine-protein consequently becomes misleading and its use should be discontinued.

We have not dealt with coenzyme II in our experiments, but its general behaviour and mode of action are so similar to those of cozymase that there is little doubt that the same considerations apply to it.

[1] This criterion was suggested to us by Prof. Keilin.

By no means all anaerobic dehydrogenases depend for their power of reducing methylene blue etc. on one or other of these two coenzymes. Quite a number are altogether independent of these coenzymes and their properties strongly suggest that they do not depend on a coenzyme at all. In this group may be included the following dehydrogenases: succinic, lactic of yeast, α-hydroxyglutaric, α-glycerophosphoric (insoluble), choline dehydrogenases and some others. These can all reduce methylene blue in the absence of cozymase and coenzyme II. The view expressed in this paper brings the coenzyme-dependent dehydrogenases more into line with these coenzyme-independent dehydrogenases, and makes it possible to consider the anaerobic dehydrogenases as one group of enzymes. The difference becomes simply one of enzyme specificity towards the H-acceptor: one group of enzymes can react directly with many acceptors, including methylene blue, quinone, alloxan and other substances; the other is more specific and cannot react directly with methylene blue or quinone, but can react with a number of other substances, including alloxan and cozymase.

On the view that the coenzymes form prosthetic groups it is of course still possible to bring the two classes of enzymes into line by assuming that the coenzyme-independent dehydrogenases contain other prosthetic groups of unknown nature. There is, however, no indication that this is the case. Attempts to decide the point are at present being made.

In conclusion we would suggest that the growing tendency to refer to dehydrogenases[1] and certain other enzymes simply as proteins is not one to be encouraged, as it tends to obscure their essentially enzymic character. No doubt enzymes are proteins, but not all proteins are enzymes, and the great bulk of the protein of the body is catalytically inactive. It is important to retain some term by which these highly specialized catalytic proteins may be distinguished from ordinary proteins; just as for example it is convenient to retain the term virus, although the viruses are also proteins.

Even in the enzymes which have prosthetic groups, such as the flavoproteins, the protein parts are by no means to be regarded as ordinary proteins. It is the protein part which determines the specificity and which contains the "active centre", or group which specifically combines with the substrate and thereby produces that profound change in its properties which we speak of as activation.

[1] The term dehydrogenase (or dehydrase) has been in regular use for a great many years in the sense of its original definition, namely to denote an enzyme which, with or without the addition of a coenzyme, brings about the dehydrogenation of its substrate by hydrogen acceptors. The term "oxidase system" has been kept for the complete system (usually containing more than one enzyme) which enables the substrate to react with O_2. For example the succinoxidase system consists of the succinic dehydrogenase, which enables succinate to reduce methylene blue or cytochrome, together with cytochrome *c* and cytochrome oxidase. Very recently however some workers have begun to use the term dehydrogenase in a quite unorthodox sense, namely to denote complete oxidase systems. For example it is applied to a system consisting of dehydrogenase + coenzyme + diaphorase, or one artificially built up from alcohol dehydrogenase + cozymase + catechol + catechol oxidase. This is calculated to cause the greatest confusion, and we suggest that the term should be retained with its original significance.

The argument that the "proteins" are not dehydrogenases because they cannot by themselves dehydrogenate substances cannot be accepted. The succinic dehydrogenase cannot dehydrogenate succinate without the addition of methylene blue or its equivalent, but it is none the less a dehydrogenase. In fact no enzyme can act in the absence of its substrate. Although the "proteins" may require the addition of coenzyme before any action occurs, they are dehydrogenases in the strictest sense, since they catalyse the dehydrogenation of their substrates by coenzyme much as the succinic dehydrogenase catalyses the dehydrogenation of succinate by methylene blue.

The prosthetic flavin group, which is common to many enzymes, merely acts as an acceptor of hydrogen from the substrate so activated. Thus the protein parts of these enzymes, and the anaerobic dehydrogenases themselves, may be regarded as analogous to the hydrolysing enzymes, which are proteins containing active centres but in all probability no prosthetic groups, as pointed out by Northrop [1935].

Summary

1. Two typical cozymase-dependent dehydrogenases, the alcohol and malic dehydrogenases of yeast, have been studied with a wider range of hydrogen acceptors than hitherto. The substances used were those previously shown to function as acceptors with the xanthine dehydrogenase.

2. The H-acceptors fall into three groups:

(*a*) those which require the presence of cozymase and flavoprotein: dyes (with certain exceptions), *m*-dinitrobenzene (probably), cytochrome etc.;

(*b*) those which require the presence of cozymase but not flavoprotein: benzoquinone, *o*-quinone, phenazine dyes, $K_3Fe(CN)_6$;

(*c*) those which do not require the presence of either cozymase or flavoprotein: alloxan, I_2, H_2O_2, dibromophenolindophenol.

Thus cozymase is not necessary for all acceptors. Cozymase is itself only one of several acceptors which react directly with the activated substrate.

3. It has been shown by two independent methods that the dehydrogenases alone (i.e. the so-called "proteins") can bring about the oxidation of their respective substrates in the complete absence of cozymase when acceptors of group (*c*) are used. Thus the "proteins" are in fact complete enzymes.

4. The relationship of dehydrogenase to coenzyme is that of enzyme to substrate rather than that of protein to prosthetic group. A number of arguments is given against the view that the coenzymes are to be regarded as the prosthetic groups of the dehydrogenases, and it is suggested that the conception of "pyridine-proteins" is misleading and should be abandoned.

References

Behrend & Zieger (1915). *Liebigs Ann.* **410**, 337.
Bernheim (1938). *J. biol. Chem.* **123**, 741.
Breusch (1939). *Biochem. J.* **33**, 1757.
Dewan & Green (1937). *Biochem. J.* **31**, 1074.
Dickens & McIlwain (1938). *Biochem. J.* **32**, 1615.
Dixon (1926). *Biochem. J.* **20**, 703.
Edson (1935). *Biochem. J.* **29**, 2082.
Green, Dewan & Leloir (1937). *Biochem. J.* **31**, 934.
Haas (1937). *Biochem. Z.* **290**, 291.
Hill & Michaelis (1933). *Science*, **78**, 485.
Kubowitz (1937). *Biochem. Z.* **293**, 308.
Liebig & Wöhler (1841). *Liebigs Ann.* **38**, 357.
Lipschitz (1921). *Pflügers Arch.* **191**, 1.
Negelein & Wulff (1937). *Biochem. Z.* **293**, 351.
Northrop (1935). *Biol. Rev.* **10**, 263.
Ogston & Green (1935). *Biochem. J.* **29**, 1983.
Ostern (1933). *Hoppe-Seyl. Z.* **218**, 160.
Quastel & Wheatley (1938). *Biochem. J.* **32**, 936.
Richardson & Cannan (1929). *Biochem. J.* **23**, 68.
Schlieper (1845). *Liebigs Ann.* **55**, 251.
Warburg & Christian (1938). *Biochem. Z.* **298**, 150.
Wood (1906). *J. chem. Soc.* **89**, 1831.
Zerfas & Dixon (1940). *Biochem. J.* **34**, 365.

52. THE PRODUCTION OF AMINES BY BACTERIA

1. THE DECARBOXYLATION OF AMINO-ACIDS BY STRAINS OF *BACTERIUM COLI*

BY ERNEST FREDERICK GALE[1]

From the Biochemical Laboratory, Cambridge

(*Received 29 January 1940*)

ACKERMANN [1910; 1911], in work on bacterial putrefaction, showed that amines can be formed from certain amino-acids by mixed cultures of organisms. His method was to inoculate a synthetic medium, containing salts, peptone, glucose and an amino-acid, with decomposing pancreas and then after an incubation of some weeks to isolate the amine from this medium. Thus he demonstrated the formation of putrescine and δ-aminovaleric acid from arginine, of histamine, cadaverine, γ-aminobutyric acid and β-alanine from their corresponding amino-acids. This work is typical of the early investigations on bacterial amine formation [Ellinger, 1900; Abderhalden *et al.* 1913] showing the formation of such substances without giving information as to the processes, organisms or enzymes involved.

Later work has been concerned with the amines formed by pure strains of identified organisms, in particular those isolated from the intestine. Thus Berthelot & Bertrand [1911; 1912] isolated a "*Bacillus aminophilus intestinalis*" which proved capable of the formation of histamine, tryptamine and tyramine. Strains of *Bact. coli* and *Proteus vulgaris* form tyramine from tyrosine [Sasaki, 1914] and *iso*amylamine from leucine [Arai, 1921]. The formation of putrescine from arginine by *Bact. coli* has been reported by Akasi [1938] and Hirai [1936], while the formation of histamine from histidine has been the subject of many papers [Mellanby & Twort, 1912; Kendall & Gebauer, 1930; Hirai, 1933; Matsuda, 1933]. The technique in most of this work is the inoculation of a synthetic medium containing salts, the amino-acid in question and either glycerol or carbohydrate, with a pure strain of the organism. This is followed by a lengthy incubation and chemical isolation of the amine produced. This method is usually not quantitative and the long incubation may entail considerable variations in the enzymic make-up of the organisms as the constitution of the medium alters.

Koessler & Hanke [1919] studied the formation of histamine by a "colon bacillus" isolated from a case of cystitis and used an extraction method to remove histamine from the culture medium, followed by colorimetric estimation. They showed that whenever the amine is produced, the medium first becomes distinctly acid and that "histamine is never formed except in the presence of an easily available source of C such as glycerol or glucose". This conclusion was later [Hanke & Koessler, 1922: 1924] found to apply to many strains of "colon bacilli" isolated from faeces. Eggerth *et al.* [1939] improved the method of extraction of histamine from culture media and Eggerth [1939] investigated the histamine production by many strains of several intestinal species. The organisms were grown in various determined media containing inorganic salts, glucose and,

[1] Senior Student of the Royal Commission for the Exhibition of 1851.

in some cases, asparagine or peptone to assist growth. In favourable conditions, histamine production began within 24 hr. and continued for 4 or 5 days. Experiments in which the *p*H of the medium was adjusted during growth showed that histamine is produced most rapidly, in most cases, between *p*H 5·0 and 5·5 and not at all at reactions more alkaline than *p*H 6·5. The results reveal the large number of intestinal organisms capable of producing histamine, but as they deal with organisms growing in culture, do not indicate the exact enzymic conditions necessary for the reaction or the extent to which other amino-acids are attacked to form amines under similar conditions.

Work of the nature quoted has shown that (*a*) carbohydrate is necessary for amine formation during growth and that (*b*) the medium becomes acid during the incubation. Such experiments however cannot show whether the action of carbohydrate is essential as such, or is due to the acid formed from it by fermentation, or whether the action of such acid is to cause the organism to form amines as a defensive "buffer reaction" as suggested by Hanke & Koessler [1924] or if its effect is due to the properties of the enzymes involved. Virtanen & Laine [1937] and Virtanen *et al.* [1938] have shown the formation of β-alanine and γ-aminobutyric acid from aspartic and glutamic acids respectively by the legume bacteria, and have also shown the quantitative formation of cadaverine from lysine by *Bact. coli*. They have avoided the difficulty of interpreting experiments in which growth is occurring by using thick suspensions of the organism and incubating these in a non-nutrient medium containing phosphate and lysine only. The long period of incubation used (3 weeks), however, does not allow of exact conditions being determined. The cadaverine produced was estimated by isolation as the picrate.

The present series of investigations has been planned to answer, by combination of washed suspension, manometric and chemical techniques, the questions: (1) what amines are produced by certain intestinal bacteria? (2) what enzymes are involved and what are their properties? (3) what environmental conditions are required for the production of such enzymes? and (4) to what extent can such findings be generalized? This communication deals with the findings with a representative selection of *Bact. coli* strains and it is intended to follow this, if circumstances permit, with the results of similar investigations with other groups of intestinal bacteria.

Preliminary work. In the above literature, *Bact. coli* is often cited as being an amine former and it is noticeable that in every case where it has proved to be such, the strains have been grown in a medium containing either glucose or glycerol. In the work of this department on bacterial deamination [Stephenson & Gale, 1937; Gale & Stephenson, 1938] it has been consistently noted that the formation of the deaminases of *Bact. coli*, which have optimum *p*H values between 7·5 and 8·0, is almost completely inhibited when glucose is present in the growth medium. It was thought that, under such conditions, the organism might proceed to attack the amino-acids of the growth medium in another manner—namely, by decarboxylation without deamination. Accordingly, the stock strain of *Bact. coli* was grown in glucose broth and the activities of the washed suspension towards various amino-acids investigated as set out below. At first the experiments were carried out in slightly acid buffers at *p*H 6·0–6·5, with completely negative results. When the experiments were repeated at *p*H 5·5, a small evolution of gas was obtained in the presence of arginine; this gas proved to be CO_2 and no NH_3 was liberated under the conditions in which it was evolved. When the reaction with arginine was studied over the *p*H range 5·0–2·0, it immediately became obvious that previous work was probably negative as the

experiments were carried out at too alkaline a reaction. Hence the work was repeated with the results presented below.

Methods. In order to obtain as wide a range of activities with each organism as possible, the cultures were grown in a tryptic digest of casein as a basal medium providing a fairly complete mixture of amino-acids. The temperature and time of incubation are dealt with below. At the end of the incubation the organisms were centrifuged out of the growth medium, washed in distilled water and then made up into suspension in distilled water. The dry weight of organism per ml. suspension was determined in each case by a photoelectric turbidimeter [Clifton *et al.* 1935] previously calibrated against various strains of *Bact. coli*. The evolution of CO_2 from amino-acids under the action of the washed suspensions was studied in Warburg manometers. As the reactions all occur at *p*H values more acid than 5·5, the CO_2 is quickly liberated into the gas phase and the pressure increase can be followed directly. The following quantities were used: in the main compartment, 1 ml. 0·05 *M* phthalate buffer, 0·5 ml. water (1 ml. in controls), 1 ml. washed suspension of organism and, in the side bulb, 0·5 ml. *M*/30 solution of the amino-acid under investigation. Thus complete decarboxylation of the amino-acid liberated 373 μl. CO_2. The manometers were filled before use with N_2 and the amino-acid solution tipped into the main compartment of the manometer after equilibration in the thermostat. The various buffers used were made from 0·2 *M* potassium hydrogen phthalate by the addition of standard acid or alkali and the *p*H checked potentiometrically every few days. The isolation of the amines from large scale experiments is described later.

Strains of Bact. coli *used*

Bact. coli (stock). The stock strain in use in this laboratory which has been kept on tryptic broth agar for several years.

Bact. coli Esch. National Institute of Type Cultures no. 86; "original *Escherichia coli*".

Bact. coli Sheffield. Isolated from horse faeces and given to us by the University of Sheffield Bact. Dept.

Bact. coli Dunn. Freshly isolated by us from a case of cystitis.

Bact. coli 201. Strain isolated from water in 1922.

Bact. coli 210 and 211. Haemolytic urinary strains, isolated 1936.

Bact. coli 216. Faecal *coli* isolated from Cam water.

Bact. coli 217. Wilson's Type I.

Bact. coli Faecal A, B, C. Faecal *coli* (Type I) freshly isolated.

Intermediate A, B. Freshly isolated coliform intermediate, Type I.

The organisms 201, 210, 211, 216 and 217 were given to us by Dr Spooner, and the freshly isolated coliforms A, B, C, Int. A and Int. B, by Dr Carruthers of the Cambridge University Pathology Dept., to whom the author is grateful for their assistance.

Decarboxylation of amino-acids by Bact. coli (*stock*)

The preliminary experiments indicated that washed suspensions of *Bact. coli* (stock), grown in 2% glucose broth, attacked arginine when incubated with it anaerobically in the presence of phthalate buffer at *p*H 5·5, causing an evolution of CO_2 ($Q_{CO_2}=7=\mu$l. CO_2 evolved/mg. dry weight of bacteria/hr.). Manometers were therefore set up containing similar amounts of organism, etc., but in the presence of buffers ranging from *p*H 2·0 to 6·0 with intervals of 0·5 *p*H unit. The resulting activities (expressed as Q_{CO_2}) are shown in Fig. 1. The optimum *p*H for CO_2 evolution is about 4·0 ($Q_{CO_2}=83$). The experimental temperature

was 30°. Repetition of the experiment at *p*H 4·0 with 0·3 ml. 20% NaOH in the centre cup of the manometer gave no gas output, showing that the evolved gas is CO_2. In each case, at the completion of the experiment, the contents of the cups were tested for NH_3 formation by distillation in Conway vessels followed by nesslerization, with completely negative results. Controls with organism alone gave no CO_2 output or NH_3 formation at *p*H 4·0 under the experimental conditions. Thus the washed suspension decarboxylates arginine without coincident deamination under these conditions.

Having established the conditions with arginine, the experiments were repeated with other amino-acids. The action of washed suspensions at *p*H 4·0 and 5·0 was tested upon the following amino-acids:

Glycine.	*l*-(+)Aspartic acid.
dl-Alanine.	*l*-(−)Proline.
dl-Valine.	*dl*-Serine.
l-(−)Leucine.	*l*-(−)Cysteine.
l-(−)Phenylalanine.	*l*-(+)Arginine.
l-(−)Tyrosine.	*l*-(+)Ornithine.
l-(−)Tryptophan.	*l*-(−)Histidine.
l-(+)Glutamic acid.	*l*-(+)Lysine.

The amino-acid solutions were prepared from the products of Hoffmann La Roche, Ltd.

The Q_{CO_2} of the organism alone was negligible but in no case has a Q_{CO_2} of <1 been taken as significant. Evolution of CO_2 was obtained with glutamic acid, histidine, lysine and ornithine in addition to arginine: in no case was there any coincident deamination. In the positive cases, the optimum *p*H was determined in a manner similar to that used for arginine and the results are also shown in Fig. 1.

Glutamic acid is decarboxylated optimally at *p*H 4·0 when $Q_{CO_2}=62$; the optimum is sharp, for the activity is almost completely inhibited at *p*H 3·0 on the acid side and at *p*H 5·0 on the alkaline. The activities towards the other three amino-acids are much lower than towards arginine and glutamic acid and do not permit of such an accurate determination of the *p*H optimum. Lysine and histidine have Q_{CO_2} of the order 8–15 between *p*H 4·0 and 4·5, while with ornithine the rate is scarcely significant, showing a Q_{CO_2} of 2 at about *p*H 4·5. In order to obtain a linear decarboxylation in these experiments the following quantities of organism were used in each manometer: for arginine and glutamic acid, 5–7 mg.; for lysine and histidine, 17–20 mg.; and for ornithine about 30 mg.

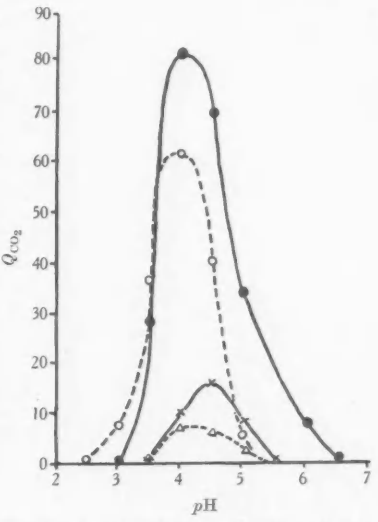


Fig. 1. Decarboxylation of amino-acids by washed suspensions of *Bact. coli* (stock): variation of activity (Q_{CO_2}) with *p*H. •—• Arginine. ○- - -○ Glutamic acid. ×—× Lysine. △- -△ Histidine.

dry weight of organism. The Q_{CO_2} figures are calculated from the linear rate of CO_2 evolution for the first 20–30 min. after the amino-acid has been tipped in. All activities in the following experiments were determined at the optimum *p*H for the decarboxylase in question.

The properties of the reactions and the enzymes involved are discussed in detail later: the demonstration of decarboxylation without deamination would indicate that amines are being formed under the above experimental conditions and this is confirmed by the isolation of the products, but before dealing with this it is necessary to establish the optimal conditions for the production of such substances by the organism.

Temperature conditions. Early experiments carried out at 37° showed that although the decarboxylation reactions occurred at this temperature with an initially high Q_{CO_2}, the rate rapidly fell off and it seemed probable that this was due to the thermolability of the enzymes involved. To investigate this, manometers were set up in four thermostats set at 19, 26, 31 and 38·5° respectively. The contents of the cups were as usual and the effect investigated for the decarboxylation of arginine and glutamic acid (dry weight of organism = 4·9 mg.), lysine and histidine (dry weight of organism = 19·2 mg.), each at its optimum *p*H value. The washed suspension was prepared from a culture grown for 18 hr. in 2% glucose broth at 27°. After the addition of the amino-acid in each case, readings were taken at 10 min. intervals for 3 hr. Fig. 2 shows the course of the

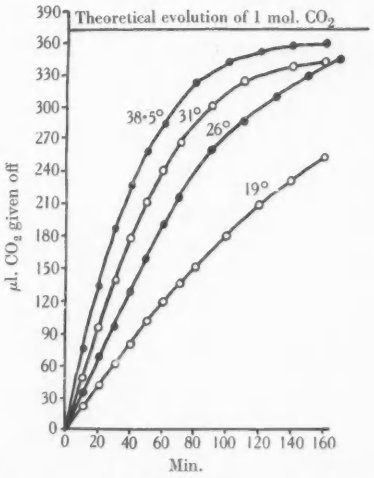


Fig. 2. Decarboxylation of arginine by washed suspensions of *Bact. coli* (stock) (4·9 mg.) at *p*H 4·0 and various temperatures.

decarboxylation of arginine at the various temperatures: the reaction is not linear but becomes more nearly linear the lower the temperature. At all temperatures the decarboxylation ceases at about 360 μl.: 373 μl. correspond to the removal of one molecule of CO_2 from the arginine molecule.

The results obtained with the other decarboxylation reactions are similar except in the case of glutamic acid. Fig. 3 shows the variation with temperature

of the initial Q_{CO_2}, measured over the first 10 min. of the reaction. The decarboxylations of arginine, lysine and histidine give an initial Q_{CO_2} increasing steadily with temperature, the temperature coefficients over the range 20–30° being: arginine 2; lysine 6·5; histidine 6. The reaction with glutamic acid is, however, much more sensitive to temperature, temperatures above 26° being inhibitory so that the rate does not increase above 26° and falls rapidly above 31°. Apart

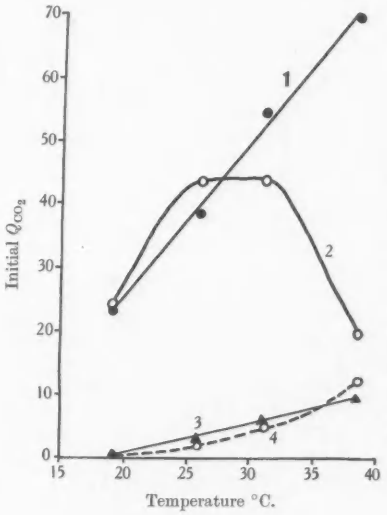


Fig. 3.

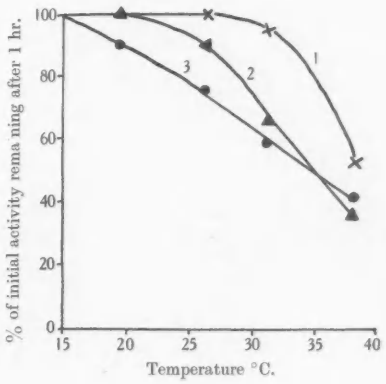


Fig. 4.

Fig. 3. Effect of temperature on initial velocity (Q_{CO_2}) of decarboxylation of amino-acids by washed suspensions of *Bact. coli* (stock). 1, Arginine. 2, Glutamic acid. 3, Histidine. 4, Lysine.

Fig. 4. Effect of temperature on amino-acid decarboxylases of *Bact. coli* (stock). 1, Lysine. 2, Histidine. 3, Arginine.

from this case, the effect of temperature is seen not in the initial Q_{CO_2} but in the diminution of the rate with time. Fig. 4 shows the percentage activities remaining after 1 hr. reaction at the various temperatures. Thus to maintain the full activity of the washed suspension towards most of the amino-acids in question, it is necessary to carry out the experiments below 20°. However, owing to the low activities at such temperatures, it has been found more convenient to use an experimental temperature of 30°, planning the quantities so that the reaction is completed in about 1 hr. and measuring rates over the early linear portion of the curve.

Growth conditions

Effect of growth temperature. Since temperature has such a marked effect upon the activities of the washed suspension, it is to be expected that the temperature at which the culture is grown will affect the activities of the organism. Table 1 shows the activities at 30° and optimum *p*H towards various amino-acids of washed suspensions of *Bact. coli* (stock) and *Bact. coli Esch.*

Table 1. *Effect of growth temperature on decarboxylase activity*

Organism	Substrate	Culture grown in 2% glucose broth: (*a*) at 27° Q_{CO_2}	(*b*) at 37° Q_{CO_2}
Bact. coli (stock)	Arginine	72	37
	Glutamic acid	65	66
	Lysine	21	10
	Histidine	9	3
Bact. coli Esch.	Arginine	240	128
	Lysine	210	68
	Histidine	18	12
	Ornithine	90	8

All activities determined at the optimum *p*H and 30°.

prepared from cultures grown in 2% glucose broth for 18 hr. at (*a*) 27° and (*b*) 37°. The higher growth temperature results in significantly smaller activities and all cultures were grown in future at 27°.

Age of culture. In studies on certain deaminases, the activity of the organism has been found to vary enormously with the time for which the culture was incubated before harvesting [Gale & Stephenson, 1938]. To investigate whether such variations occurred with the decarboxylases, 2 l. of 2% glucose broth were inoculated with a 20 hr. culture of *Bact. coli* (stock), incubated at 27° and samples

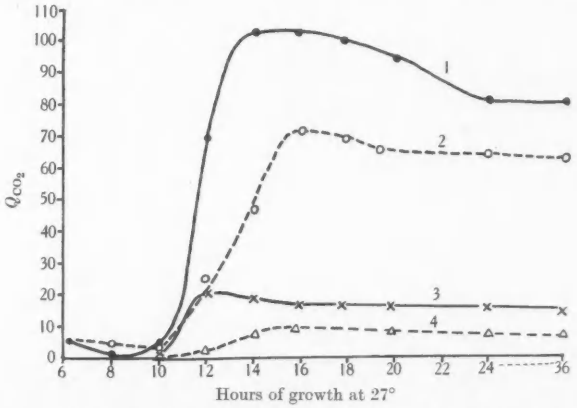


Fig. 5. Variation of decarboxylase activity (Q_{CO_2}) with "age of culture": *Bact. coli* (stock) grown in 2% glucose broth at 25°; growth ceases 15–17th hr. 1, Arginine. 2, Glutamic acid. 3, Lysine. 4, Histidine.

withdrawn at 2 hr. intervals from the 6th hr., when turbidity was first visible, up to the 20th hr. and then at 24 and 36 hr. The samples were withdrawn with sterile precautions, the turbidity and *p*H measured, the organisms centrifuged out of the culture, washed and made up into washed suspension. The activities of the suspensions toward arginine, glutamic acid, lysine and histidine were then determined in each case under optimal conditions and the results are shown in Fig. 5. Early cultures have little activity, the activity increasing rapidly between 11th–14th hr. of growth, becoming maximal from 14th to 16th hr.

when active cell division has finished. After this the activity declines slowly as the culture ages. There is no abrupt alteration in *p*H during the growth period but the *p*H falls slowly until it eventually stabilizes at about 5 after 48 hr. The variation in activity with age of culture is similar to that found with aspartase [Gale, 1938] which was shown to be due to some undefined change in the medium brought about by the metabolic activities of the organism. As in that case, the maximum activity coincides with the cessation of active cell division, and in all cases mentioned later where there is doubt about the correct growth period, the culture was harvested at the time when the turbidity ceased to increase—so that all cultures are harvested in approximately the same biological phase.

Nature and presence of carbohydrate in growth medium. It has been noted that whenever bacteria have been shown to produce amines in culture media, some form of carbohydrate or glycerol has been present and in all the experiments, so far quoted, the organisms have been grown in 2% glucose broth. Having determined the optimal conditions for the investigation of decarboxylation by the washed suspension, it is now necessary to find what part, if any, the carbohydrate in the growth medium plays. To do this, cultures of the organism were grown in the following ways: on the surface of broth agar in Roux bottles; in liquid broth under strictly anaerobic conditions; and in broth in flasks with the addition, separately, of glycerol, glucose, fructose, mannose and galactose to a final concentration of 2% in each case. In certain cases powdered chalk was included in the glucose medium. In all cases the inoculated medium was incubated at 27° until the turbidity ceased to increase (16–20 hr.), the final *p*H in the medium measured potentiometrically and washed suspensions prepared from the harvested organism. The activities towards arginine, glutamic acid, lysine and histidine were then determined under optimal conditions. Fig. 6 sets out diagrammatically the mean results obtained in each case.

When the organism is grown on the surface of broth agar, it has negligible decarboxylase activity. Strictly anaerobic conditions result in a small activity while the presence of glycerol in the growth medium produces the first marked decarboxylase activity. The presence of glucose in the growth medium results in the highest activity in each case, the effect being diminished by the presence of chalk. The presence of fructose, mannose or galactose has approximately the same effect as that of glucose, but not quite so marked in some cases, galactose having only a small enhancing effect in the case of arginine decarboxylase.

Fig. 6 shows that glucose produces an increase of activity that cannot be accounted for by the anaerobiosis produced by fermentation gases and that the effect is by no means specific for glucose as this can be replaced by other sugars and, to a smaller extent, by glycerol. When the final *p*H in the medium is considered in each case, it appears that there is a correlation between the *p*H of the medium and the activity of the organism, which accounts for the smaller effect of glycerol and the effect of the presence of chalk in the glucose medium.

Accordingly, a series of flasks of plain broth was set up and their *p*H values adjusted roughly to 8·5, 7·5, 7, 6, 5 and 4 respectively. The media were inoculated with a 20 hr. broth culture of the stock strain and incubated at 27°. The cultures were harvested in each case when the turbidity ceased to increase and the final *p*H determined. The periods of incubation varied from 16 hr. at *p*H 7 to 22 hr. at *p*H 5, the total crop of organism was less the further the growth *p*H diverged from 7·0 to 7·5, and at *p*H 4·0, although a slight turbidity was produced in 30 hr., insufficient organism was obtained to harvest. The activities of the washed suspensions were determined as usual and the results are shown in Fig. 7. The production of the four decarboxylases increases with decrease of growth *p*H,

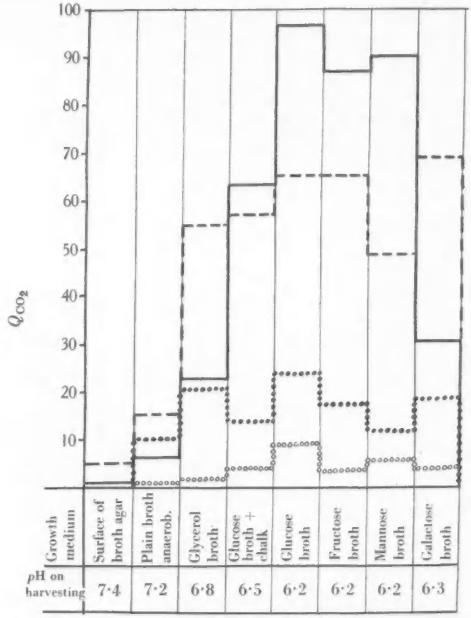


Fig. 6. Variation of decarboxylase activities of washed suspensions of *Bact. coli* (stock) with constitution of growth medium. All cultures grown at 27°. Activities determined at 30° and at optimum *p*H. —— Arginine. - - - - Glutamic acid. • • • Lysine. o o o Histidine.

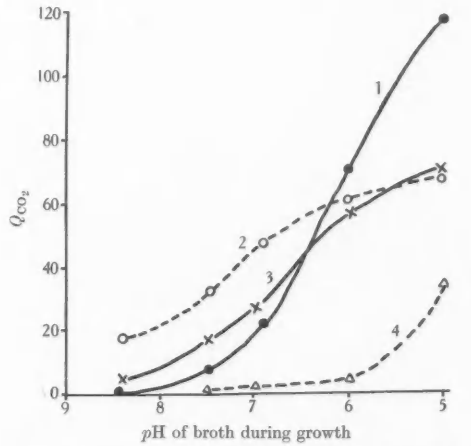


Fig. 7. Variation of decarboxylase activity (Q_{CO_2}) of washed suspensions of *Bact. coli* (stock) with the *p*H of the plain broth in which the organism was grown. 1, Arginine. 2, Glutamic acid. 3, Lysine. 4, Histidine.

that of the enzymes for arginine, lysine and histidine being negligible at *p*H 8 but reaching values higher than any obtained in the carbohydrate media when grown at *p*H 5. If the activities shown in Fig. 6 are superimposed on those in Fig. 7 it can be seen that the activities in the various media (Fig. 6) are fully accounted for by the *p*H reached in those media during growth. Thus the presence of carbohydrate in the medium has no specific effect but acts through the production of acid from it by fermentation, the organism actually producing more decarboxylase in a non-carbohydrate medium at an acid reaction. In many of the general experiments quoted later the organism was grown in 2% glucose broth as by this means a larger crop is obtained than by growing in an acid medium.

The findings so far are related to the decarboxylases of arginine, glutamic acid, lysine and histidine. *Bact. coli* (stock) also probably decarboxylates ornithine but at a rate too slow to allow of detailed investigation; this amino-acid is however attacked rapidly by *Bact. coli Esch.* Hence the properties of this organism will next be considered.

Decarboxylation of amino-acids by Bact. coli Esch.

Investigation of the decarboxylase activities of washed suspensions of this organism grown in 2% glucose broth at 27° for 18 hr. showed that, of the list

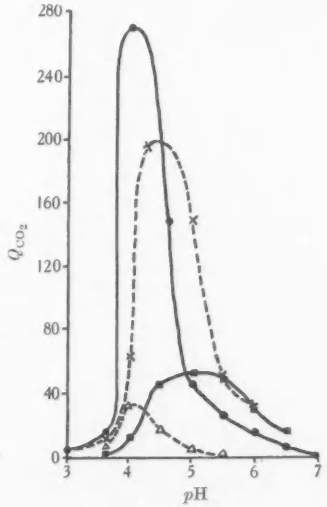


Fig. 8.

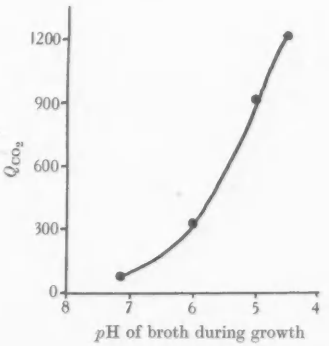


Fig. 9.

Fig. 8. Decarboxylation of amino-acids by washed suspensions of *Bact. coli Esch.*: variation of activity (Q_{CO_2}) with *p*H. •—• Arginine. × - - - - × Lysine. △ - - - - △ Histidine. ■—■ Ornithine.

Fig. 9. Variation of ornithine decarboxylase activity (Q_{CO_2}) of washed suspensions of *Bact. coli Esch.* with the *p*H of the broth in which the organism was grown.

of amino-acids set out above, the only ones attacked significantly are arginine, lysine, ornithine and histidine. Fig. 8 shows the *p*H-activity curve obtained for these amino-acids with the washed suspension. The activities are in general much higher than those shown by *Bact. coli* (stock), and this is possibly due to

the fact that *Esch.* is a more rapid acid former, the final *p*H in glucose medium after 18 hr. growth being 5·2 compared with 6·2 for the stock strain. Glutamic acid is not attacked. Fig. 8 shows an exceptionally sharp optimum at *p*H 4·0 for the arginine decarboxylase; at 4·5–4·7 for lysine; at 4·0 for histidine, and a less sharply defined optimum at about *p*H 5·0 for the ornithine enzyme. The Q_{CO_2} values obtained at the optimum *p*H and 30° are: arginine 272; lysine 198; ornithine 48 and histidine 33.

Fig. 9 shows the increase in Q_{CO_2} for the ornithine decarboxylase of the washed suspension with the decrease in *p*H of the plain broth growth medium (cf. Fig. 7 for stock). The value when grown in broth at *p*H 7·2 is 47 and this increases very rapidly to the extremely high figure of 1200 when the growth *p*H is 4·5. This enzyme thus gives a remarkable demonstration of the effect of the growth *p*H. It is noticeable in this case that the glucose growth medium which reaches a final *p*H of 5·2 gives rise to an organism which has an ornithine decarboxylase activity of only 48—in this case, the glucose medium is apparently inhibitory to the enzyme formation. This point will be discussed later.

General properties of the amino-acid decarboxylases

Quantitative nature of the decarboxylation. Fig. 10 shows the course of the decarboxylation of the five amino-acids so far studied by washed suspensions of either *Bact. coli* (stock) or *Esch.* under optimal conditions. The theoretical

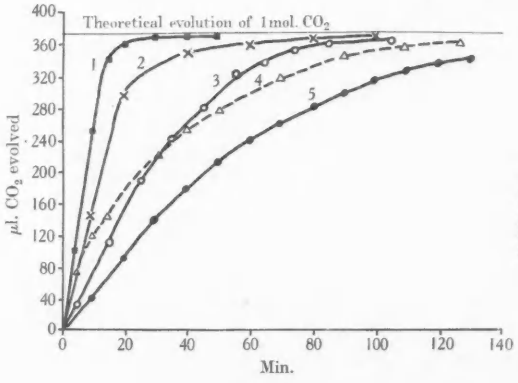


Fig. 10.

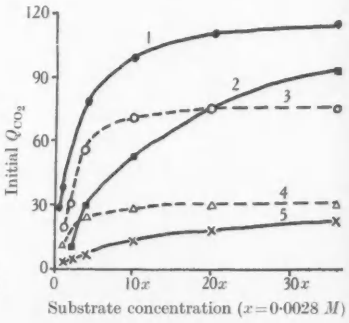


Fig. 11.

Fig. 10. Quantitative decarboxylation of amino-acids: action of washed suspensions on 0·5 ml. *M*/30 amino-acid. 1, Ornithine: 1·9 mg. *Bact. coli Esch.* (culture from broth at *p*H 5). 2, Lysine: 11·4 mg. *Bact. coli Esch.* (2% glucose broth culture). 3, Glutamic acid: 8·0 mg. *Bact. coli* (stock) (2% glucose broth culture). 4, Histidine: 30·0 mg. *Bact. coli Esch.* (2% glucose broth culture). 5, Arginine: 4·9 mg. *Bact. coli Esch.* (2% glucose broth culture).

Fig. 11. Variation of initial rate of decarboxylation (Q_{CO_2}) with concentration of substrate. 1, Arginine (stock). 2, Ornithine (*Esch.*) 3, Glutamic acid (stock). 4, Histidine (*Esch.*). 5, Lysine (stock).

evolution of CO_2 corresponding to the removal of 1 mol. of CO_2 from the molecule of amino-acid is 373 μl. and it can be seen that in every case the decarboxylation ceases within a few μl. of this figure. Thus the reaction is quantitative.

Affinities of the decarboxylases. Fig. 11 represents the variation of initial Q_{CO_2} with the substrate concentration for the five enzymes in *Bact. coli* (stock) and *Esch.* The approximate concentration for half maximum reaction velocity in each case is given in Table 2.

Table 2. *Apparent Michaelis constant for amino-acid decarboxylases*

Substrate	Organism	Michaelis constant
Arginine	*Bact. coli* (stock)	0·00056 *M*
Glutamic acid	*Bact. coli* (stock)	0·00056 *M*
Lysine	*Bact. coli* (stock)	0·0028 *M*
Histidine	*Bact. coli Esch.*	0·00075 *M*
Ornithine	*Bact. coli Esch.*	0·003 *M*

Action of glucose on activities of washed suspension. The action of glucose in the growth medium has been stated to be due to the acids produced from it by fermentation. Table 3 shows the effect of the presence of glucose during the decarboxylation by washed suspensions prepared from cultures in 2% glucose broth. The presence of glucose during the decarboxylation by washed suspensions therefore has no significant effect.

Table 3. *Decarboxylation of amino-acids in presence of 1% glucose*

The values for Q_{CO_2} are corrected for the glucose blank.

Organism	Substrate	Q_{CO_2} Alone	Q_{CO_2} +Glucose
Bact. coli (stock)	Arginine	67	56
	Glutamic acid	50	45
	Lysine	6	6
	Histidine	6	6
Bact. coli Esch.	Arginine	253	247
	Lysine	244	261
	Histidine	16	17
	Ornithine	54	63
	Glucose at *p*H 4·0	—	0

Presence of oxygen. Two sets of manometers were set up to test the decarboxylations carried out by the stock organism under optimal conditions, one set filled with N_2 and the other with air. No significant difference in the rate of CO_2 evolution occurred and no deamination could be shown in the aerobic experiments—probably since the experimental *p*H diverges widely from the *p*H optimum for the deaminases [Stephenson & Gale, 1937]. To avoid any strain differences in this matter, however, all other experiments were carried out anaerobically.

Coenzyme effect. In previous investigations on deaminases [Gale & Stephenson, 1938; Gale, 1938] evidence has been obtained from the loss in activity of washed suspensions on standing under various conditions that has led to the postulation of coenzymes required by these enzymes. Similar experiments have been carried out with the decarboxylases: as a source of possible coenzymes a suspension of baker's yeast in phosphate buffer (*p*H 6) was prepared and boiled for 10 min., 0·5 ml. of this preparation was then added to the contents of the Warburg cups involved. The activities of a washed suspension of *Bact. coli* (stock) towards arginine, glutamic acid, lysine and histidine were determined (*a*) immediately after preparation of the washed suspension and under the usual optimal conditions; (*b*) as (*a*) but with the addition of the boiled yeast preparation; (*c*) after

the washed suspension had been incubated (in water suspension) for 4 hr. at 27°, and (*d*) as (*c*) with the addition of yeast. The resulting values of Q_{CO_2} in each case are set out in Table 4.

Table 4. *Effect of* (*i*) *addition of boiled yeast,* (*ii*) *incubation at 27°, on the decarboxylase activities of washed suspensions of* Bact. coli (*stock*)

For key to column headings see text.

	Q_{CO_2}			
Substrate	(*a*)	(*b*)	(*c*)	(*d*)
Arginine	69	82	52	86
Glutamic acid	57	66	33	80
Lysine	18	23	14	18
Histidine	4	4·5	3	4·5
Boiled yeast control	0	0	0	0

Comparison of (*c*) with (*a*) shows that the incubation of the washed suspension in water leads to a loss of activity although this is not very marked. As a result of this, the activities of all other suspensions were determined, unless otherwise stated, as soon after preparation as possible. Comparison of (*d*) with (*c*) and (*a*) shows that this lost activity can be fully restored by the presence of boiled yeast, the activity returning to a figure higher than that shown by the original washed suspension alone—but which is shown by the initial suspension in the presence of the yeast (*b*). Thus there is some factor present in boiled yeast which increases the decarboxylase activities of the organism and which brings about restoration of the activity lost by the washed suspension on standing. The factor cannot be replaced by a pure preparation of cocarboxylase (aneurin pyrophosphate).

Isolation of the amines found

The general technique evolved for the isolation of the amines formed by the bacterial decarboxylation of amino-acids is as follows: from the knowledge obtained by the manometric experiments, quantities are worked out for repeating the manometric experiment on a scale sufficiently large to decarboxylate an amount of amino-acid to completion and to provide 100–200 mg. of the amine picrate on isolation. The actual quantities vary with the activity of the organism and examples are set out below. The solutions are put in a "Kreb's pot", gassed with N_2 and then incubated at 30°; at the same time a control is carried out manometrically and the incubation is continued until the manometer indicates that the decarboxylation has reached completion. At the end of the incubation, the organism is removed by filtration through a Seitz pad and the filtrate then evaporated to dryness *in vacuo*. The dry residue is extracted several times with absolute alcohol made slightly alkaline by the addition of a drop of 10 % NaOH. To obtain quantitative recovery it is necessary to carry out the extraction in a reflux apparatus some seven or eight times, but in the majority of isolations described below four ½-hr. extractions were found to yield about 80 % of theory. The alcoholic extracts are filtered, made just acid to bromothymol blue with acetic acid and saturated alcoholic $HgCl_2$ then added until precipitation ceases. After standing overnight, the clear supernatant is retested against alcoholic $HgCl_2$. The precipitate is then centrifuged down, resuspended in about 10 ml. water and decomposed with H_2S. The filtrate is boiled to remove H_2S, adjusted to *p*H 6 and the theoretical amount of 10 % picric acid in methyl alcohol then added. The solution is brought to the boil and then allowed to cool slowly, when crystals of the amine picrate are precipitated. These are recrystallized from

water until their melting points agree with the values given in the literature as below. The analyses are by Weiler, the amine being isolated as the picrate in each case.

Isolation of agmatine from arginine. Quantities: 40 ml. washed suspension *Bact. coli* (stock) prepared from 1 l. 2 % glucose broth culture grown for 18 hr. Total dry weight of organism 340–350 mg. 40 ml. 0·05 *M* phthalate buffer *p*H 4·0. 40 ml. *M*/60 *l*-(+)arginine (neutral). Yield: 256 mg. orange needles; on heating these crystals soften and appear to melt at 228–229°, decomposing sharply at 235–236°. Found: C, 34.83 %; H, 3·47 %; N, 24·03 %. Calc.: C, 34·69 %; H, 3·41 %; N, 23·81 %.

Isolation of cadaverine from lysine. Quantities: 20 ml. washed suspension *Bact. coli* (stock) as above. 20 ml. 0·05 *M* phthalate buffer *p*H 4·5. 20 ml. *M*/60 *l*-(+)lysine dihydrochloride (neutral). Yield: 158 mg. deep yellow needles: M.P. 221°. Found: C, 36·35 %; H, 3·63 %; N, 19·65 %. Calc.: C, 36·42 %; H, 3·57 %; N, 20·0 %.

Isolation of histamine from histidine. Quantities: 20 ml. washed suspension *Bact. coli Esch.* prepared from 1 l. 2 % glucose broth culture grown for 18 hr. Total dry weight of organism 580 mg. 20 ml. 0·05 *M* phthalate buffer *p*H 4·0. 20 ml. *M*/60 *l*-(−)histidine monohydrochloride (neutral). Yield: 120 mg. orange-yellow needles: M.P. 235° (uncorr.), 240° (corr.). Found: C, 36·28 %; H, 2·81 %; N, 21·8 %. Calc.: C, 35·97 %; H, 2·64 %; N, 22·2 %.

Isolation of putrescine from ornithine. Quantities: 40 ml. washed suspension *Bact. coli Esch.* prepared from a culture grown in plain broth at *p*H 5·0 for 20 hr. Total dry weight of organism 50 mg. 40 ml. 0·05 *M* phthalate buffer *p*H 5·0. 40 ml. *M*/60 *l*-(+)ornithine dihydrochloride (neutral). Yield: 163 mg. light yellow needles: on heating these do not melt but decompose with blackening at 248–250°. Found: C, 35·20 %; H, 3·65 %; N, 20·35 %. Calc.: C, 35·03 %; H, 3·65 %; N, 20·44 %.

Isolation of γ-aminobutyric acid from glutamic acid. Quantities: 40 ml. washed suspension *Bact. coli* (stock) prepared from 2 % glucose broth culture as above. 40 ml. 0·05 *M* phthalate buffer *p*H 4·0. 40 ml. *M*/60 *l*-(+)Na glutamate. The usual technique was followed as far as alcoholic extraction of dry residue: then $AgNO_3$ was added until precipitation ceased. Alcoholic extraction was continued until the filtered extract gave no precipitate with $AgNO_3$. Centrifuged down and dried *in vacuo*; yield 136 mg. Found: 51·1 % Ag. Calc. for Ag aminobutyrate: 51·4 %.

For the isolation of the γ-aminobutyric acid as such, the above experiment was repeated on a 10 times larger scale using the organism harvested from 10 l. of 2 % glucose broth. The alcoholic extract was prepared as before and the acid precipitated as its Hg salt by the addition of saturated alcoholic Na acetate and $HgCl_2$ [Ackermann, 1910]. After standing overnight the precipitate was centrifuged down, redissolved in alcohol, filtered and reprecipitated. The centrifuged precipitate was washed in alcohol, suspended in water, decomposed with H_2S and the filtrate reduced *in vacuo* to small bulk. This was then placed in a vacuum desiccator and allowed to evaporate to dryness; very hygroscopic crystals appearing in the last stages. To obtain the free acid Ag_2O, 50 % in excess of theory, was added to the aqueous solution: after shaking in the cold for some time, the AgCl was filtered off, the filtrate concentrated to small bulk and left standing in a desiccator containing alcohol. Crystals separated in a few days: M.P. 192–194° (uncorr.). The M.P. of γ-aminobutyric acid is variously given in the literature as 183–202° while that of α-aminobutyric acid is 283°. Further distinctions between the two aminobutyric acids are discussed later.

Decarboxylation of amino-acids by various strains of Bact. coli

The properties of the decarboxylases of arginine, ornithine, lysine, histidine and glutamic acid have been studied for those enzymes appearing in *Bact. coli* strains stock and *Esch.* These findings are now applied to other strains of the coliform group. The procedure was as follows: each organism was first grown in 2% glucose broth and the activities of its washed suspension were then investigated at *p*H 4·0 (and in some cases at *p*H 5·0) under standard conditions for the decarboxylation of the sixteen amino-acids listed early in this paper. In those cases where a positive evolution of CO_2 was obtained, unaccompanied by deamination, not explicable by the organism blank and having a $Q_{CO_2} > 1$, the reaction was then studied over the *p*H range 3·0–6·0 and the optimum *p*H so determined. A list of the optimum activities with the various strains is given in Table 5.

Table 5. *Decarboxylase activities of* Bact. coli *strains grown for 18 hr. in 2% glucose broth at 27°*

Activities expressed as Q_{CO_2} determined with washed suspensions at the optimum *p*H and 30°.

		Q_{CO_2}				
No.	Organism	Arginine	Histidine	Lysine	Ornithine	Glutamic acid
1	*Bact. coli* stock	90*	*10**	*19**	2	67*
2	*Bact. coli Esch.*	272*	33*	198*	*48**	– †
3	*Bact. coli Sheff.*	*146**	14*	59*	*4**	63
4	*Bact. coli Dunn*	5	2	206*	1	– †
5	*Bact. coli* 201	– †	*2*	121*	–†	152*
6	*Bact. coli* 211	*158**	*12*	53	*4*	107
7	*Bact. coli* 210	98*	–†	*24*	?	86
8	*Bact. coli* 216	95*	4	44*	6	130
9	*Bact. coli* 217	96*	*5*	*28*	*5**	105
10	*Bact. coli faecal* A	98*	*6*	*34**	*1**	95
11	*Bact. coli faecal* B	– †	–†	– †	5	– †
12	*Bact. coli faecal* C	116*	9	72*	*6*	82
13	*Coliform intermed.* A	*7*	*3*	4	*6*	?
14	*Coliform intermed.* B	*45**	*1*	*1*	*6*	– †
15	*Bact. formicum*	15	6	*2*	*2*	– †
16	*Bact. friedländeri*	– †	3	34	8	– †

* Indicates amine isolated and identified: the isolations of putrescine were carried out with the organism grown in broth at *p*H 5 (see Table 6). Italicized figures indicate that value is more than doubled by growth in broth at *p*H 5.

† Indicates activity $Q_{CO_2} < 1$.

In no case did the washed suspension attack any of the listed amino-acids, other than the five in Table 5, at a significant rate under the experimental conditions. For the organisms 1–6 the *p*H curve was carried out for the decarboxylation activities with each amino-acid showing a positive result, but as the optimum *p*H obtained was always the same, namely: arginine *p*H 4·0; histidine *p*H 4·0; lysine *p*H 4·5; ornithine *p*H 5·0; glutamic acid *p*H 4·0; the activities were determined in the other cases only at these optimum *p*H values. In many cases the amines were isolated as the picrates as indicated above and the picrates then identified by M.P. determination and, in representative cases, by analysis. In the case of arginine decarboxylation the isolation was carried out in all cases showing an activity > 10 owing to the confusion in the literature with regard to the product of this reaction (see later) and the product in every case was agmatine and never putrescine.

Of the 14 coliform organisms investigated, 7 attacked all five amino-acids and 11 attacked the four excluding glutamic acid: 12 decarboxylated arginine; 12 histidine; 13 lysine; 12 ornithine and 9 glutamic acid. Histidine and ornithine are attacked much more slowly than the other three amino-acids but ornithine decarboxylase is better produced under different growth conditions, as shown below.

Effect of growth p*H on the formation of the decarboxylases.* The preliminary studies with *Bact. coli* (stock) and *Esch.* showed that the activities of the organism obtained from culture in glucose broth were due to the low *p*H produced during growth by the acid formed from the glucose. Organisms grown in plain broth previously adjusted to *p*H 5·0 had higher activities than those grown in glucose broth, this effect being particularly marked in the case of ornithine decarboxylase (*Bact. coli Esch.*). To test the general application of this finding cultures of each of the other strains mentioned were grown (*a*) in plain broth at *p*H 7·2 and (*b*) in plain broth at *p*H 5·0; the activities of the washed suspension were determined as usual, and these were compared with the values obtained with the glucose broth organism. The results are set out in Table 6. As the results are best shown with ornithine as substrate, the organisms are put down in order of the activity towards ornithine of their cultures grown at *p*H 5. The organisms are also numbered to compare with Table 5 and the Q_{CO_2} values are italicized in those cases where the activity of the *p*H 5 culture is more than twice that of the glucose culture. All activities are determined under optimal conditions.

Table 6. *Effect of growth* p*H on decarboxylase activity* (Q_{CO_2})

In each case the Q_{CO_2} is given for washed suspensions prepared from cultures grown at *p*H 7·2 (7), at *p*H 5·0 (5), and in 2% glucose broth (GB).

		Final *p*H	Ornithine			Arginine			Lysine			Histidine			Glutamic acid		
No.	Organism	(GB)	7	5	GB	7	5	GB	7	5	GB	7	5	GB	7	5	GB
2	*Bact. coli Esch.*	5·25	47	*960*	48	2	338	272	53	194	198	3	26	33	—	—	1
9	*Bact. coli* 217	5·3	59	*840*	6	2	133	96	23	*113*	28	5	*17*	5	23	96	105
10	*Bact. coli faecal* A	5·1	40	*612*	1	7	83	98	6	*91*	34	—	*12*	6	23	87	95
3	*Bact. coli Sheff.*	5·15	13	*200*	3	95	*380*	146	18	76	60	3	16	14	32	54	63
13	*Coliform inter.* A	5·4	4	*47*	6	15	*149*	7	2	5	4	1	*11*	3	8	2	—
12	*Bact. coli faecal* C	5·6	2	*30*	6	4	115	116	18	110	72	1	10	9	10	99	82
14	*Coliform inter.* B	5·2	1	*29*	6	14	*135*	53	—	4	1	—	14	1	—	—	—
6	*Bact. coli* 211	5·3	—	*20*	4	28	*302*	154	29	58	53	4	*30*	12	36	72	107
15	*Bact. formicum*	5·05	—	*10*	2	5	22	15	4	*15*	2	2	6	6	—	—	—
16	*Bact. friedländeri*	5·1	—	4	8	—	*4*	—	5	51	34	—	2	3	—	—	—
8	*Bact. coli* 216	5·2	1	3	6	4	114	95	7	82	44	—	6	4	23	71	130
1	*Bact. coli* (stock)	6·25	—	2	1	8	120	90	12	*70*	20	1	*30*	10	20	66	67
7	*Bact. coli* 210	5·55	1	2	1	2	50	98	9	*102*	24	—	1	3	19	40	86
4	*Bact. coli Dunn*	5·1	—	1	2	—	5	1	22	373	206	—	1	3	—	—	—
11	*Bact. coli faecal* B	5·65	3	1	5	—	—	—	—	—	—	—	—	—	—	—	—
5	*Bact. coli* 201	5·3	—	—	—	—	—	—	56	142	121	2	*10*	2	48	81	182

The results given in Table 6 show that:

(*a*) Organisms grown at *p*H 7·2 have little decarboxylase activity.

(*b*) This activity is greatly increased by growing the organisms at *p*H 5·0 or by the presence of glucose in the growth medium.

(*c*) The increase in activity produced by the presence of glucose is fully explained by the fall in *p*H produced by the fermentation acid.

(*d*) In the case of ornithine decarboxylase the presence of glucose in the growth medium does not increase the formation of enzyme but appears to inhibit this in some cases. The effect of growth *p*H is particularly marked with this enzyme.

Specificity of the decarboxylases. Table 7, derived from Table 5, shows the distribution of the decarboxylases among various *Bact. coli* strains and associated organisms and, since no one enzyme is constantly associated with any other in this distribution, this would indicate their strict specificity.

Table 7. *Distribution of the decarboxylases*

	Substrate				
Organism	Ornithine	Arginine	Lysine	Histidine	Glutamic acid
Bact. coli 217	+	+	+	+	+
Bact. coli Esch.	+	+	+	+	−
Bact. coli 210	−	+	+	−	+
Bact. coli 201	−	−	+	+	+
Bact. friedländeri	+	−	+	+	−
Bact. coli faecal B	+	−	−	−	−

Characteristics of the amino-acid decarboxylases

Arginine decarboxylase. Optimum *p*H 4·0; temp. coeff. 2; Michaelis constant (stock) 0·00056 *M*; product—agmatine.

As far as the author knows there is no previous record of the production of agmatine from arginine by bacteria. Linneweh [1931] isolated agmatine from amongst the products of bacterial decomposition of arcaine but the records of bacterial attack of arginine give putrescine as the amine produced [Ackermann, 1910; Hirai, 1936; Akasi, 1938; etc.]. Mueller [1925] states that agmatine cannot be obtained from arginine by putrefaction. The production of putrescine from arginine in the presence of mixed cultures [Ackermann, 1910] is probably due to the presence of organisms containing arginase and, where the period of incubation is lengthy [Hirai, 1936], coliform organisms may produce that enzyme by adaptation. In the work of Akasi [1938] 21 out of 54 strains of *Bact. coli* produced putrescine when grown in a synthetic medium containing arginine in which the arginine formed the sole source of N. Using washed suspensions as described in this work, the product is agmatine which can be easily differentiated from putrescine not only by the decomposition point of its picrate but also by the colour of the crystals, since agmatine picrate forms deep orange needles, and putrescine picrate forms yellow needles. Moreover, as can be seen from Table 5, in every case the rate of decarboxylation of arginine by organisms grown in glucose broth is considerably greater than that of ornithine, so that the latter cannot be an intermediate substance in the amine formation. Growth experiments differ from washed suspension experiments in that the *p*H may vary greatly in the former and it may be that ornithine is formed from arginine during the early stages of some growth experiments while the *p*H is near neutrality, but that the enzymes responsible for such a change are inactive at *p*H 4·0, the optimum *p*H for the decarboxylation of arginine by the washed suspension.

Agmatine is reported to possess marked physiological actions of an insulin-like nature and its properties have been studied by Frank *et al.* [1926]; Frank [1927]; Hazard [1929]; etc.

Ornithine decarboxylase. Optimum *p*H 5·0; Michaelis constant (*Esch.*) 0·003 *M*; product—putrescine.

Ornithine decarboxylase formation increases rapidly with decreasing *p*H of the growth environment (Fig. 9; Table 6). It differs from the other decarboxylases studied in that the presence of glucose in the growth medium does not,

by the production of acid, lead to an increased enzyme formation (Table 6). The presence of glucose does not affect the decarboxylation of ornithine by the washed suspension—this has been shown in Table 3 and has been confirmed by experiments with *Bact. coli* 217 grown at *p*H 5 in plain broth. Thus, since glucose does not affect the enzyme once it is formed, it must have some effect on the formation of the enzyme. Table 8 shows the effects of various growth conditions on the activity of the enzyme in *Bact. coli* 217.

Table 8. *Effects of* (a) *growth* p*H*, (b) *glucose, on the ornithine decarboxylase activity of* Bact. coli *217*

Growth medium	Initial *p*H	Final *p*H	Q_{CO_2} (ornithine) (1)	(2)
Plain broth	7	7	59	75
Plain broth	5	5	840	645
2% glucose broth	7	5·3	9	6
2% glucose broth	5	4·8	142	128

The results show that not only does the presence of glucose during growth at *p*H 7, when vigorous fermentation occurs, inhibit the formation of the enzyme some 80–90%, but also when growth occurs at *p*H 5 and only slight fermentation occurs, its presence still causes an 80% reduction in the amount of enzyme formed. It would thus appear that in this case the presence of glucose inhibits the formation of the decarboxylase during growth, the effect being similar, superficially, to that noted for the deaminases [Stephenson & Gale, 1937].

Since the ornithine decarboxylase activity is so much greater in organisms grown in non-glucose media at *p*H 5 than when grown at *p*H 7, an attempt was made to increase the activity of a washed suspension, harvested from a culture grown in plain broth at *p*H 7, by incubating it at *p*H 5 either alone or in the presence of ornithine. Table 9 shows that the result was negative.

Table 9. *Attempt to increase activity of organism grown at* p*H 7*

Ornithine decarboxylase activity (Q_{CO_2}) of *Bact. coli Esch.*

	Q_{CO_2}
(*a*) Freshly prepared suspension	19
(*b*) Suspension after incubation at *p*H 7 for 3 hr. at 27°	12
(*c*) Suspension after incubation at *p*H 5 for 3 hr. at 27°	16
(*d*) Suspension as (*c*) in presence of *M*/10 ornithine	23
(*e*) Freshly prepared suspension from culture grown at *p*H 5	960

Lysine decarboxylase. Optimum *p*H 4·5; Michaelis constant (stock)—0·003 *M*; temp. coeff. 6·5; product—cadaverine.

Virtanen & Laine [1937] state that certain strains of *Bact. coli* decarboxylate lysine to give cadaverine, the optimum *p*H for the reaction being 7. Since their experiments continued over several weeks' incubation and their estimations depend on a final isolation of the cadaverine picrate, their method is not capable of exact determination of optimum conditions.

Histidine decarboxylase. Optimum *p*H 4·0; Michaelis constant (*Esch.*) 0·00075 *M*; temp. coeff. 6; product—histamine. The nature of the product has been confirmed by quantitative biological tests by Dr Feldberg of the Cambridge University Physiology Dept., to whom the author is grateful for his co-operation.

Glutamic acid decarboxylase. Optimum *p*H 4·0; Michaelis constant (stock) 0·00056 *M*; "optimum temp." 25°; product—γ-aminobutyric acid. The decarboxylation of glutamic acid ceases when 1 mol. of CO_2 has been removed from

the acid molecule. The properties of the product in the two cases studied in detail indicate that it is the γ-aminobutyric acid remaining. This is confirmed by (1) only 87 % of the amino-N is given off in the Van Slyke estimation for α-amino-groups and (2) the dissociation constants of the decarboxylation product (Fig. 12) agree with those found for γ-aminobutyric acid by Neuberger [1937].

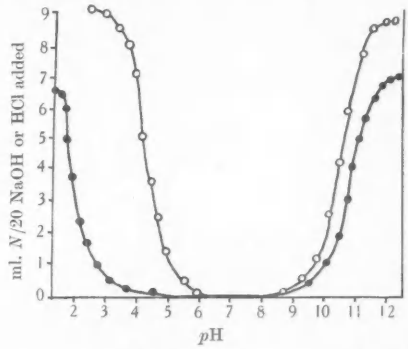


Fig. 12. Titration curve of α-aminobutyric acid •—• and product of decarboxylation of glutamic acid by *Bact. coli* (stock) o—o.

Table 10 indicates the specificity of the glutamic acid decarboxylase of *Bact. coli* (stock) (see also Table 7).

Table 10. *Specificity of glutamic acid decarboxylase*

Experiments with washed suspensions of *Bact. coli* (stock) at *p*H 4.

Substrate	Q_{CO_2}
l-Glutamic acid	58–67
l-Glutamine	25–30*
N-Acetylglutamic acid	0
l-Aspartic acid	0
l-α-Aminobutyric acid	0

* Preceded by deamidation.

Adaptive nature of the decarboxylases. Table 11 gives the activities (Q_{CO_2}) of washed suspensions of *Bact. coli Esch.* and 216 grown in the following synthetic media:

Medium A	Medium B
Stephenson's inorganic medium	Stephenson's inorganic medium
2% Na lactate	2% Na lactate
*p*H adjusted to 5·5	2% glucose
Medium C = medium A + 1% arginine	*p*H (initial) 6·8
Medium D = medium B + 1% arginine	
Medium E = medium B + 1% lysine	
Medium F = medium B + 1% glutamic acid	

Under present conditions, insufficient histidine or ornithine were available for use in growth media.

The decarboxylases are not developed in amino-acid-free media with the exception of the glutamic acid decarboxylase of 216 which would thus appear to be constitutive (in the sense used by Karstrom [1938]), although its production is increased by the presence of its substrate in the growth medium. The lysine

Table 11. *Decarboxylases of* Bact. coli *grown in various synthetic media*

Medium	Q_{CO_2} Arginine	Ornithine	Lysine	Histidine	Glutamic
Bact. coli Esch.					
A	0	1	0	0	—
B	1	2	3	0	—
C	0	—	—	—	—
D	27	—	—	—	—
E	—	—	270	—	—
Plain broth at *p*H 5	338	960	194	17	0
Bact. coli 216					
B	4	2	0	1	50
D	7	5	7	0	35
F	2	3	0	0	96
Plain broth at *p*H 5	114	3	82	6	71

decarboxylase of *Esch.* is strictly adaptive. The presence of arginine in the growth medium does lead to an increased production of the arginine decarboxylase but not to any marked extent; possibly the formation of this enzyme is dependent also on the production of some coenzyme that cannot be synthesized with ease in a simple medium. Owing to shortage of amino-acids, the results in this last section are based upon a few experiments and cannot be regarded as conclusive.

Reference to Fig. 6 shows that the organism, when grown on the surface of agar containing broth, possesses no significant decarboxylase activity. Attempts to produce arginine decarboxylase in such a washed suspension by incubating it at 27° for 4 hr. under the following conditions have proved negative:

(1) Anaerobically in phthalate buffer at *p*H 4·0.
(2) As (1) with the addition of *M*/100 arginine.
(3) As (1) with the addition of 1 % glucose.
(4) As (2) with the addition of 1 % glucose.
(5) Anaerobically in the presence of glucose at *p*H 6·0.
(6) As (5) with the addition of *M*/100 arginine.

Thus the enzyme is not produced in the non-dividing organism by incubation at the optimum *p*H with or without its substrate or in the presence of glucose, fermenting or non-fermenting.

Discussion

From the above work it would appear that *Bact. coli* produces enzymes capable of forming amines by the decarboxylation of certain amino-acids if the organism grows in the presence of the amino-acids at an acid reaction; the lower the *p*H the more enzyme being produced, within physiological limits. The enzymes once formed will act over a restricted *p*H range, being most active between *p*H 4 and 5. Hanke & Koessler [1924] obtained evidence that led to similar conclusions and wrote "the production of amines from amino-acids seems to be a protective mechanism and is resorted to when the accumulation of H ions within the organism's protoplasm is incompatible with its normal life processes. The amines can thus be thought of as reaction buffers". It must, however, also be remembered that when the reaction becomes acid, amino-acids can no longer be attacked by deamination, for the deaminases are inactivated and, at *p*H values lower than 5, carbohydrate itself is but slowly attacked; the amino-groups of the amino-acids become ionized but the —COOH groups become discharged and the production of decarboxylases may be the method

by which the organism extends its range of existence, utilizing amino-acid decarboxylation when other substrates and methods of attack are no longer available.

The extent to which conditions suitable for the production and activity of decarboxylases in bacteria in the intestine exist will depend upon the nature of the diet, presence of unabsorbed amino-acids, carbohydrate and acid-producing bacteria in the intestine. It is hoped to deal with some of these points in a later communication.

Summary

1. Washed suspensions of *Bact. coli* (stock) prepared from a culture grown in 2% glucose broth (tryptic digest of casein) decarboxylate arginine, lysine, histidine and glutamic acid to form agmatine, cadaverine, histamine and γ-aminobutyric acid respectively.

2. Washed suspensions of *Bact. coli Esch.*, similarly prepared, decarboxylate arginine, lysine, histidine and ornithine to form agmatine, cadaverine, histamine and putrescine respectively.

3. The decarboxylases have sharp *p*H-activity maxima, the optimum value in each case being: for arginine 4·0; lysine 4·5; histidine 4·0; glutamic acid 4·0 and ornithine 5·0.

4. The enzymes exhibit great sensitivity to rise of temperature and a convenient experimental temperature of 30° is used.

5. Washed suspensions prepared from organisms grown at 27° are more active than those prepared from organisms grown at 37°.

6. Young cultures have little activity, the activity rising to a maximum between the 14th and 16th hr. of incubation, after which it falls steadily to the 48th hr.

7. Experiments on the variation of growth conditions show that the presence of fermentable carbohydrate in the medium gives rise to an organism possessing far greater activity than one grown in a non-carbohydrate medium at *p*H 7. The action of the carbohydrate is due to the action of the acid produced from it by fermentation.

8. The production of the decarboxylases in the organism varies with the reaction of the growth medium: the lower the *p*H, the higher the activity of the resulting culture in washed suspension. This effect is particularly marked with ornithine decarboxylase, the culture grown at *p*H 5 having 20–100 times the activity of one grown at *p*H 7.

9. The decarboxylation is quantitative.

10. The affinities of the decarboxylases of *Bact. coli* (stock) and *Esch.* are determined.

11. Neither the presence of O_2 nor of glucose affects the course of the decarboxylations carried out by washed suspensions of *Bact. coli* (stock) and *Esch.* under optimal experimental conditions.

12. Evidence is given that the decarboxylases require a coenzyme, which is not aneurin pyrophosphate.

13. In every case the amine has been isolated and identified.

14. Investigation of the decarboxylation activities of 14 strains of coliform organisms shows that 12 strains decarboxylate arginine to form agmatine; 12 histidine to histamine; 13 lysine to cadaverine; 12 ornithine to putrescine, and 9 decarboxylate glutamic acid.

15. In all cases the optimum *p*H of the decarboxylases proved constant and equal to the values given in (3).

16. In all cases (*a*) organisms grown at *p*H 7 have little decarboxylase activity; (*b*) this activity is greatly increased by growing the organisms at *p*H 5 or by the presence of glucose in the growth medium; (*c*) the increase in activity produced by the presence of glucose is fully explained by the fall in *p*H during growth due to fermentation acids.

17. In contradistinction to the other four decarboxylases, the production of ornithine decarboxylase is inhibited by the presence of glucose in the growth medium but, as usual, greatly increased by a low growth *p*H in a non-carbohydrate medium.

18. The distribution of the decarboxylases indicates that each enzyme is specific for the decarboxylation of one amino-acid.

19. The characteristics of the individual enzymes are discussed.

20. The enzymes are not produced if the organisms are grown in an amino-acid-free medium.

21. In no case studied was any of the natural amino-acids, other than the five mentioned, decarboxylated at a significant rate ($Q_{CO_2} > 1$), by washed suspensions under the experimental conditions described.

The author wishes to thank Mr R. Davies for his assistance in the confirmatory investigations at the end of this paper, also Dr M. Stephenson and Prof. Sir Frederick Hopkins for their interest and encouragement throughout the course of this work. He is indebted to the Royal Commissioners for the Exhibition of 1851 for the Senior Studentship during the tenure of which this work has been carried out.

REFERENCES

Abderhalden, Fromme & Hirsch (1913). *Hoppe-Seyl. Z.* **85**, 131.
Ackermann (1910). *Hoppe-Seyl. Z.* **65**, 504; **69**, 273.
—— (1911). *Z. Biol.* **56**, 88.
Akasi (1938). *Act. Sch. med. Univ. Kioto*, **22**, 433.
Arai (1921). *Biochem. Z.* **122**, 251.
Berthelot & Bertrand (1911). *C.R. Acad. Sci., Paris*, **154**, 1643.
—— —— (1912). *C.R. Acad. Sci., Paris*, **154**, 1826.
Clifton, Mueller & Rogers (1935). *J. Immunol.* **29**, 377.
Eggerth (1939). *J. Bact.* **37**, 205.
—— Littwin & Deutsch (1939). *J. Bact.* **37**, 187.
Ellinger (1900). *Hoppe-Seyl. Z.* **29**, 334.
Frank (1927). *Naturwissenschaften*, **15**, 213.
—— Morgan & Wagner (1926). *Klin. Wschr.* **5**, 2100.
Gale (1938). *Biochem. J.* **32**, 1583.
—— & Stephenson (1938). *Biochem. J.* **32**, 392.
Hanke & Koessler (1922). *J. biol. Chem.* **50**, 131.
—— —— (1924). *J. biol. Chem.* **59**, 835, 867, 855.
Hazard (1929). *J. Pharm. Chim., Paris*, **9**, 371.
Hirai (1933). *Biochem. Z.* **267**, 1.
—— (1936). *Biochem. Z.* **283**, 390.
Karstrom (1938). *Ergebn. Enzymforsch.* **7**, 350.
Kendall & Gebauer (1930). *J. infect. Dis.* **47**, 261.
Koessler & Hanke (1919). *J. biol. Chem.* **39**, 497, 521, 539.
Linneweh (1931). *Hoppe-Seyl. Z.* **200**, 115; **202**, 1; **205**, 126.
Matsuda (1933). *Nagasaki Igakkwai Zassi*, **11**, 821.
Mellanby & Twort (1912). *J. physiol.* **45**, 53.
Mueller (1925). *Biochem. Z.* **83**, 320.
Neuberger (1937). *Proc. roy. Soc.* A, **158**, 68.
Sasaki (1914). *Biochem. Z.* **59**, 429.
Stephenson & Gale (1937). *Biochem. J.* **31**, 1316.
Virtanen & Laine (1937). *Enzymologia*, **3**, 266.
—— Rintala & Laine (1938). *Nature*, **142**, 674.

53. BENZEDRINE (β-PHENYL*ISO*PROPYLAMINE) AND BRAIN METABOLISM

By P. J. G. MANN and J. H. QUASTEL

From the Biochemical Laboratory, Cardiff City Mental Hospital

(*Received 8 January 1940*)

It has been shown by Quastel & Wheatley [1933] that the addition of a variety of amines, including tyramine and *iso*amylamine, to brain tissue respiring in a glucose-phosphate medium results in a fall in the rate of O_2 uptake by the tissue. The oxidations of glucose, lactic acid, pyruvic acid and glutamic acid by brain were found to be markedly inhibited by exposure of the tissue to such amines. Succinic acid oxidation is not impaired, however, except in the case of brain tissue exposed to tyramine. In this case a large fall in the rate of succinic acid oxidation takes place.

A study of the mechanism by which several aliphatic amines which retard brain respiration undergo breakdown in isolated tissue resulted in the finding [Pugh & Quastel, 1937, 1] that brain contains an enzyme capable of oxidizing aliphatic amines with the liberation of NH_3. Typical instances are butylamine, *iso*amylamine and heptylamine. The oxidase in question was found to be quite distinct from amino-acid oxidase.

Subsequent work carried out independently by Pugh & Quastel [1937, 2] and by Blaschko *et al.* [1937, 1] showed that the aliphatic amine oxidase was identical with tyramine oxidase and the enzyme is now known as amine oxidase. The former authors proved by analysis of the reaction products formed when *iso*amylamine and butylamine are oxidized by liver extracts that the following transformations take place:

$$R.CH_2NH_2 + O_2 + H_2O = R.CHO + NH_3 + H_2O_2,$$
$$2R.CHO + H_2O = R.CH_2OH + R.COOH.$$

Richter [1937; see also Kohn, 1937] showed that the enzyme oxidizes phenylethylamine, tyramine and arterenol to form NH_3; adrenaline, epinine and sympatol to produce methylamine; and alkamine and hordenine to give ethylamine and dimethylamine respectively. The corresponding aldehydes are also formed; these were isolated as the dinitrophenylhydrazones. The general reaction was represented as follows:

$$R.CH_2\overset{+}{N}HR_2^1 + \tfrac{1}{2}O_2 = R.CHO + \overset{+}{N}H_2R_2^1,$$

where R^1 = hydrogen or an alkyl group. Blaschko *et al.* examined a large number of amines and showed that only compounds containing an amino group at the end of a hydrocarbon chain are oxidized. When the α carbon atom was substituted as in the cases of *iso*propylamine and ephedrine no attack by the enzyme took place. It was suggested that the free hydrocarbon chain was an essential part of the substrate since molecules such as cadaverine and histamine are not attacked.

When intact tissue slices (brain, liver, kidney) are examined under suitable physiological conditions it is found that the addition of tyramine or β-indolethylamine to the medium in which the slices are respiring does not result in

an increased O_2 uptake, although there is no doubt that the amine is undergoing oxidation. This is shown by the liberation of NH_3 and of the corresponding aldehyde [Pugh & Quastel, 1937, 2]. The rate of O_2 uptake of the tissue slices falls, the rate of fall increasing with time; this effect is much more marked with brain cortex slices than with liver or kidney cortex slices. To show an increased O_2 consumption of the intact tissues in presence of amines it is necessary to inhibit the normal respiration of the tissues by the addition of cyanide or of arsenite.[1] Amine oxidase is unaffected by cyanide or arsenite and in the presence of the latter molecules it is possible to show that tyramine oxidation by intact tissues results in the absorption of one atom of O_2 and the formation of one molecule of NH_3 for each molecule of amine oxidized. The same applies to the oxidation of indolethylamine, although here there is evidence that in the presence of arsenite more than one atom of O_2 is consumed for each molecule of amine decomposed. It is obvious that for an investigation of the properties of amine oxidase it is necessary to use tissue extracts or preparations where other oxidizing systems have been eliminated as far as possible.

Similar conclusions to these were arrived at by Blaschko *et al.* [1937, 2] in their studies of adrenaline inactivation by tissues.

Competition for amine oxidase takes place between various amines. Thus it was found by Pugh & Quastel [1937, 2] that competition between the following amines occurs: tyramine, *iso*amylamine, β-indolethylamine and β-phenylethylamine. Blaschko *et al.* [1937, 1] showed that competition occurs between tyramine, adrenaline, *iso*amylamine and β-indolethylamine. The latter authors also showed that *l*-ephedrine, triethylamine, tri*iso*amylamine, hordenine—amines which are either slowly or not attacked by amine oxidase—have an affinity for the enzyme and inhibit the oxidation of amines which are attacked by the oxidase. Kohn [1937] arrived at similar results.

Benzedrine (β-phenyl*iso*propylamine) is either very feebly or not at all attacked by amine oxidase [Pugh & Quastel, Blaschko *et al.*]. It has nevertheless a high affinity for this enzyme and is a powerful inhibitor of amine oxidation.

It is the purpose of this communication to show that the presence of benzedrine at low concentration markedly stimulates the respiration of brain *in vitro* whether this is examined in the form of intact tissue slices or as a mince so long as the respiration takes place in the presence of a variety of amines, and that this stimulation is directly related to the affinity of benzedrine for amine oxidase.

Technique

O_2 uptakes were determined with the Warburg respiratory apparatus. The methods for the preparation and examination of tissue slices are described in an earlier paper [Pugh & Quastel, 1937, 2] and will not be further commented upon here.

Effects of benzedrine on the respiration of brain tissue in presence of tyramine, indolethylamine and *iso*amylamine

Brain cortex slices

The presence of tyramine, indolethylamine, *iso*amylamine or certain other amines results in a progressive fall in the respiration of brain cortex slices respiring in a glucose-phosphate-Locke medium at 37° [Quastel & Wheatley, 1933; Pugh & Quastel, 1937, 1, 2]. Benzedrine at relatively high concentrations also reduces

[1] Or of a high phosphate concentration [Philpot, 1937].

the respiration of brain cortex slices examined in a glucose medium, but it has little or no effect at small concentrations (e.g. $10^{-4}M$).

When benzedrine[1] at small concentrations is added to a glucose medium containing tyramine, indolethylamine or *iso*amylamine the fall in the rate of O_2 uptake takes place much less rapidly than in the absence of the benzedrine. Typical results relating to rat brain cortex slices are shown in Table 1. In the case of tyramine at a concentration of $7{\cdot}7 \times 10^{-3}M$ the respiration falls from a value of $Q_{O_2}=9{\cdot}3$ during the 1st hour of the experiment, to 4·8, during the 2nd hour, and to 2·6 during the 3rd hour. When benzedrine at a concentration of $4{\cdot}3 \times 10^{-4}M$ which has little or no inhibitory action on the respiration of brain cortex slices is added to the glucose medium containing tyramine $7{\cdot}7 \times 10^{-3}M$ the fall in respiration is given by $Q_{O_2}=9{\cdot}7$ during the 1st hour, 8·4 during the 2nd hour and 6·0 during the 3rd hour. The benzedrine effects a partial neutralization of the inhibition of respiration of brain cortex slices brought about by the presence of tyramine. The effect may be observed similarly with the inhibitions produced by *iso*amylamine and indolethylamine. In the latter case the inhibitory action of $0{\cdot}33 \times 10^{-3}M$ indolethylamine on rat brain cortex respiration was almost completely neutralized by the presence of $0{\cdot}43 \times 10^{-3}M$ benzedrine.

It is evident that the presence of benzedrine has the effect of stimulating the O_2 uptake of brain cortex slices when these respire in media containing amines such as tyramine, indolethylamine or *iso*amylamine. The stimulant action of benzedrine, so long as it is not used at such high concentrations (e.g. $4 \times 10^{-3}M$) that it itself exercises inhibitory effects, is inversely proportional to the concentration of tyramine or indolethylamine.

Table 1

Effects of benzedrine ($4{\cdot}3 \times 10^{-4}M$) on brain cortex respiration in presence of amines. Glucose-phosphate-Locke. O_2. 37°.

		Q_{O_2}					
		1st hour		2nd hour		3rd hour	
Amine	M conc.	Benzedrine absent	Benzedrine present	Benzedrine absent	Benzedrine present	Benzedrine absent	Benzedrine present
Nil	—	12·7	11·2	10·9	10·1	9·7	9·7
Tyramine	$7{\cdot}7 \times 10^{-3}$	9·3	9·7	4·8	8·4	2·6	6·0
	$1{\cdot}9 \times 10^{-3}$	9·7	9·7	5·2	8·6	3·0	6·0
β-Indolethylamine	$0{\cdot}67 \times 10^{-3}$	10·4	12·1	6·8	10·6	3·0	6·8
	$0{\cdot}33 \times 10^{-3}$	10·2	11·2	8·3	10·3	5·2	8·4
*iso*Amylamine	$1{\cdot}67 \times 10^{-3}$	10·5	10·7	7·6	9·2	4·9	7·6

Table 2

Effects of benzedrine on brain cortex respiration in presence of tyramine. Glucose-phosphate-Locke. O_2. 37°.

		Q_{O_2}		
Tyramine M conc.	Benzedrine M conc.	1st hour	2nd hour	3rd hour
Nil	Nil	11·4	10·1	9·0
$1{\cdot}9 \times 10^{-3}$	Nil	9·6	5·3	3·1
$1{\cdot}9 \times 10^{-3}$	$1{\cdot}4 \times 10^{-4}$	10·1	8·5	6·3
$5{\cdot}7 \times 10^{-3}$	Nil	8·8	4·9	2·7
$5{\cdot}7 \times 10^{-3}$	$1{\cdot}4 \times 10^{-4}$	11·0	6·3	2·9

This is seen in the results quoted in Tables 1 and 2.

[1] As the sulphate.

Minced tissue

The effect of benzedrine in decreasing the fall of respiration of brain tissue when this is exposed to tyramine, etc., may also be seen very clearly with minced brain tissue. Typical results obtained with a suspension of minced rat brain tissue in saline (1·3 g. brain in 7 ml. saline) are shown in Table 3; respiration was allowed to occur in a glucose-phosphate-saline medium in the presence of air.

Table 3

O_2 uptakes (μl.) of 1 ml. minced rat brain tissue at 37° in glucose (0·01 *M*)-phosphate (0·033 *M*; *p*H 7·4)-saline in the presence of tyramine and various concentrations of benzedrine. Tyramine conc. = $1{\cdot}9 \times 10^{-3}$ *M*.

Time	No amine present	Tyramine alone present	Tyramine + benzedrine Benzedrine concentrations			
			$7{\cdot}1 \times 10^{-5}$ *M*	$1{\cdot}4 \times 10^{-4}$ *M*	$2{\cdot}8 \times 10^{-4}$ *M*	$7{\cdot}1 \times 10^{-4}$ *M*
During 1st hour	304·3	240·9	234·7	236·8	233·1	238·2
During 2nd hour	249·7	145·2	156·1	173·7	186·2	192·8
During 3rd hour	205·8	74·9	109·9	128·3	162·7	180·2

It will be seen that the neutralizing action of benzedrine increases with its concentration.

Moreover, it will be noted that using a concentration of tyramine of $1{\cdot}9 \times 10^{-3}$ *M* the presence of so low a concentration of benzedrine as $7{\cdot}1 \times 10^{-5}$ *M* effects a partial neutralization of the inhibitory action of the tyramine.

The latter concentration corresponds to a concentration of β-phenyl*iso*-propylamine of about 1 mg. per 100 ml. Doubtless smaller concentrations of benzedrine would be equally effective in partially neutralizing the inhibitory effects of amines such as tyramine when these are present in very low concentrations.

Another method of observing the action of benzedrine on amine inhibition of brain respiration is to expose the minced brain tissue to mixtures of benzedrine and toxic amines for an arbitrary period after which glucose is added to the system. O_2 uptakes are now recorded. The experiments can be carried out conveniently in the Warburg apparatus, glucose being placed in the side tube of the manometer flask. Minced brain tissue is placed in a phosphate-Locke medium containing tyramine or mixtures of tyramine and benzedrine and allowed to incubate at 37° in air, the manometric apparatus being allowed to shake in the usual way for 2 hr. The levels of the fluid in the manometer are read after opening and reclosing the taps of the flask and the glucose in the side tube is tipped into the body of the flask. The rates of O_2 uptake are now read for 1 hr. Typical results obtained with minced guinea-pig brain suspension (3 g. brain in 15 ml. Locke solution) are shown in Table 4. It will be seen that when benzedrine is present in the medium the addition of tyramine brings about

Table 4

O_2 uptakes (μl.) in 1 hr. at 37° by 2 ml. minced guinea-pig brain tissue in a glucose (0·01 *M*)-phosphate (0·033 *M*; *p*H 7·4)-Locke medium after exposure for 2 hr. in the absence of glucose to tyramine and to a mixture of tyramine and benzedrine.

Amine present	μl. O_2
Nil	238·7
Benzedrine ($1{\cdot}4 \times 10^{-3}$ *M*)	177·7
Tyramine ($1{\cdot}9 \times 10^{-3}$ *M*)	90·1
Benzedrine ($1{\cdot}4 \times 10^{-3}$ *M*) + tyramine ($1{\cdot}9 \times 10^{-3}$ *M*)	170·8

little or no fall in the brain respiration. In another experiment carried out in a similar manner an O_2 uptake of 71·1 μl. found after exposure to tyramine ($1{\cdot}9 \times 10^{-3} M$) alone was increased to 196·3 μl. by the addition to the medium of benzedrine ($0{\cdot}7 \times 10^{-4} M$).

Thus the effect of adding benzedrine to brain tissue consuming O_2 in the presence of glucose and of tyramine is greatly to stimulate the O_2 uptake.

Succinic acid oxidation by brain in presence of tyramine and of mixtures of tyramine and benzedrine

It was noticed by Quastel & Wheatley [1933] that the presence of tyramine greatly inhibited succinate oxidation by brain tissue and that tyramine was unique, among the amines investigated, in this respect.

It may be shown that benzedrine neutralizes this inhibitory action of tyramine. This fact is most conveniently demonstrated by exposing brain tissue aerobically to tyramine and to a mixture of tyramine and benzedrine for 2 hr. at 37° in the absence of other substrates, adding sodium succinate solution and measuring the subsequent rate of O_2 uptake. Typical results are shown in Table 5, in which values of O_2 uptake obtained with a minced rat brain suspension (1·4 g. brain suspended in 7 ml. saline) are recorded.

Table 5

O_2 uptakes (μl.) in 1 hr. by 1 ml. minced rat brain suspension in a succinate ($0{\cdot}013 M$)-phosphate ($0{\cdot}033 M$; *p*H 7·4)-saline medium after exposure for 2 hr. in the absence of succinate to tyramine and to a mixture of tyramine and benzedrine. Air. 37°.

Amine present	μl. O_2
Nil	215·1
Tyramine ($1{\cdot}9 \times 10^{-3} M$)	112·2
Tyramine ($1{\cdot}9 \times 10^{-3} M$) + benzedrine ($4{\cdot}3 \times 10^{-4} M$)	174·1

Mechanism of action of benzedrine

If amines such as tyramine, β-indolethylamine or *iso*amylamine exert their inhibitory effects on glucose oxidation by brain by combination with some essential respiratory catalyst, it follows that benzedrine at concentrations which have no inhibitory effects on glucose oxidation can have no affinity for the respiratory catalyst in question. Hence it cannot displace the inhibitory amines from this catalyst or neutralize their inhibitory actions. Since, however, the evidence already submitted shows that the inhibitory amines and benzedrine do compete in such a way that the presence of the latter molecule neutralizes the development of an inhibition of respiration, it follows that the free amines themselves at the concentrations in question are not wholly responsible for the inhibitions. It is more likely that inhibitory agents are developed as a result of metabolism of the amines and that the benzedrine exerts its effect by retarding the development of such agents. Evidence in favour of this view is given by the fact that the inhibitions exerted by tyramine, etc., increase markedly with time (see Table 1, and Pugh & Quastel, 1937, 2) pointing to the gradual development of an inhibitory agent. That this agent is the aldehyde[1] corresponding to the inhibitory amine is made likely by the following evidence:

1. Brain contains an amine oxidase capable of oxidizing tyramine, β-indolethylamine and *iso*amylamine to the corresponding aldehydes.

[1] Or a substance formed as a result of further metabolism of the aldehyde.

2. Those amines which are not attacked by amine oxidase have little or no inhibitory effects on the respiration of brain in presence of glucose. Such amines, for instance, are cadaverine, putrescine and histamine [Quastel & Wheatley, 1933], which suffer no oxidation by amine oxidase [Blaschko *et al.* 1937, 1; Pugh & Quastel, 1937, 2]. Again benzedrine which is not oxidized by this enzyme has no inhibitory action on brain respiration at concentrations which will show effective neutralizing powers.

3. Aldehyde formation can be shown to take place gradually when brain slices or suspensions respire in presence of the inhibitory amines. The development of the aldehyde, as indicated by the dinitrophenylhydrazine-alkali test [Pugh & Quastel, 1937, 2], is much decreased by the presence of those concentrations of benzedrine which are effective in neutralizing the inhibitory action of the amines.

4. Aldehydes corresponding to, or of a similar structure to, those formed by *iso*amylamine and tyramine, exert considerable inhibitory effects on the respiration of brain in presence of glucose and these effects are not neutralized by the addition of benzedrine to the system.

Such aldehydes exert their inhibitory effects immediately in contrast to the corresponding amines whose effects develop relatively slowly with time.

The inhibitory effects of *p*-hydroxybenzaldehyde and of *iso*valeric aldehyde on rat brain cortex respiration in a glucose-phosphate-Locke medium and the lack of neutralizing action of benzedrine will be seen in the results given in Table 6.

Table 6

Effects of aldehydes on respiration of rat brain cortex slices in presence and absence of benzedrine. Glucose-phosphate-Locke. O_2. Benzedrine $= 4{\cdot}3 \times 10^{-4} M$.

		Q_{O_2}					
		1st hour		2nd hour		3rd hour	
Aldehyde	*M* conc.	Benzedrine absent	Benzedrine present	Benzedrine absent	Benzedrine present	Benzedrine absent	Benzedrine present
p-Hydroxybenzaldehyde	$2{\cdot}7 \times 10^{-3}$	9·6	8·8	7·3	7·0	4·9	5·2
*iso*Valeric aldehyde	$4{\cdot}7 \times 10^{-3}$	6·8	7·4	4·6	5·2	2·6	3·4
Nil	—	12·7	11·2	10·9	10·1	9·7	9·7

*iso*Valeric aldehyde and *p*-hydroxybenzaldehyde inhibit the respiration of minced brain tissue in a glucose-phosphate medium their toxicities increasing with their concentrations. Typical results are shown in Tables 7 and 8. In these experiments rat brain suspensions (1·5 g. brain suspended in 7 ml. saline) was employed. The lack of neutralizing effect of benzedrine is shown in Table 9. The toxicity of *iso*valeric aldehyde is perhaps lower than would have been anticipated from that of *iso*amylamine, but the question of the relative rates of penetration into the cell has to be borne in mind, as well as the fact that the amine *per se* may exert some inhibitory influence at the concentrations used.

The conclusion[1] that benzedrine exerts its stimulating action on the O_2 uptake of brain when this respires in presence of certain amines by diminishing the rate of formation of toxic aldehydes or of toxic products formed from such aldehydes is supported by the following evidence which shows that succinate

[1] A somewhat analogous case in which the presence of uric acid may accelerate oxygen uptake in the xanthine oxidase system has been noted by Harrison [1932].

Table 7

O_2 uptakes (μl.) by 1 ml. rat brain suspension in glucose (0·01 M)-phosphate (0·033 M; pH 7·4)-saline in the presence of various quantities of *iso*valeric aldehyde. Air. 37°.

*iso*Valeric aldehyde conc.	O_2		
	1st hour	2nd hour	3rd hour
Nil	277·6	219·2	186·4
10^{-3} M	244·2	191·9	169·3
3×10^{-3} M	198·5	156·1	140·8
6×10^{-3} M	128·3	94·8	79·5
10^{-2} M	82·9	47·8	29·0

Table 8

O_2 uptakes (μl.) by 1 ml. minced rat brain suspension in glucose (0·01 M)-phosphate (0·033 M; pH 7·4)-saline in presence of various quantities of p-hydroxybenzaldehyde. Air. 37°.

p-Hydroxybenzaldehyde conc.	μl. O_2 uptake	
	1st hour	2nd hour
Nil	253·5	211·9
$2{\cdot}67 \times 10^{-3}$ M	223·5	181·9
$5{\cdot}33 \times 10^{-3}$ M	193·7	138·6

Table 9

As in Table 7 but with the addition of various concentrations of benzedrine.

Conc. *iso*valeric aldehyde	Conc. benzedrine	μl. O_2 uptake	
		1st hour	2nd hour
6×10^{-3} M	Nil	125·4	102·2
6×10^{-3} M	$1{\cdot}4 \times 10^{-4}$ M	120·3	109·3
6×10^{-3} M	$5{\cdot}6 \times 10^{-4}$ M	99·5	86·7
Nil	Nil	253·5	211·9

oxidation by brain is inhibited by the aldehyde corresponding to tyramine (i.e. p-hydroxyphenylacetaldehyde).

It is of importance to mention at this point that benzedrine, in common with a number of other amines, e.g. mezcaline, will bring about inhibitions of brain respiration when used at relatively high concentrations. Since there is no evidence that benzedrine suffers oxidation by the brain at these concentrations it is clear that the inhibitions in question cannot be caused by oxidation products of benzedrine. It seems more likely that these inhibitions may be related to the presence of the non-polar phenyl*iso*propyl group acting in a manner similar to such groups in the narcotics. A discussion of this type of inhibition is, however, not relevant to the subject of this paper, for only concentrations of benzedrine are being considered which do not themselves markedly affect the respiration of brain *in vitro*.

Effect of semicarbazide on the oxidation of succinate by brain in presence of tyramine

The addition of semicarbazide to brain tissue suspended in a phosphate-saline medium containing tyramine results in a complete neutralization of the latter's inhibitory effects on succinate oxidation. Typical findings are shown in Table 10. In the experiment whose results are recorded in this table minced rat brain (1·3 g. suspended in 7 ml. saline) was exposed to tyramine and to mixtures of tyramine and semicarbazide for 2 hr. at 37° before the addition of succinate. Recordings of O_2 uptake were made immediately after this addition.

Table 10

As in Table 5 with the exception that semicarbazide is substituted for benzedrine.

Substrates present	μl. O_2 uptake
Succinate (0·033 *M*)	332·9
Succinate (0·033 *M*) + tyramine (0·0019 *M*)	126·9
Succinate (0·033 *M*) + tyramine (0·0019 *M*) + semicarbazide (0·013 *M*)	309·3
Succinate (0·033 *M*) + tyramine (0·0019 *M*) + semicarbazide (0·033 *M*)	335·3
Succinate (0·033 *M*) + semicarbazide (0·033 *M*)	339·3

This result is convincing evidence that an aldehyde, or a product of further change of an aldehyde, derived from tyramine is responsible for the inhibition of succinate oxidation by this amine in presence of brain tissue.

Tyramine and succinate oxidation by tissues

Further evidence in support of the above conclusion may be obtained from the following observations.

When succinate is oxidized by brain tissue anaerobically by ferricyanide the presence of tyramine has no inhibiting effect whatever on the rate of oxidation. On the other hand, if brain tissue is incubated aerobically for 2 hr. in the presence of tyramine and, after this period, succinate is added to the system, the subsequent rate of anaerobic oxidation of succinate by ferricyanide is greatly decreased from the normal. These results are shown in Table 11. The technique of measuring the rate of anaerobic oxidation of succinate manometrically has been explained in detail by Quastel & Wheatley [1938] and this technique was followed in the experiments recorded in Table 11. The rate of CO_2 formation is a measure

Table 11

Rat brain (1·3 g.) was minced and suspended in Locke solution. This was centrifuged and the deposit made up to 8 ml. with Locke solution. 1 ml. suspension was placed in 0·025 *M* bicarbonate in presence and absence of 0·0019 *M* tyramine. This was exposed either (*a*) to 95% O_2 + 5% CO_2 or (*b*) to 95% N_2 + 5% CO_2 for 2 hr. at 37°. The gas in the former case was then changed to 95% N_2 + 5% CO_2. Succinate (0·033 *M*) was added, followed by 0·2 ml. ferricyanide solution.

	μ CO_2 formed
Exp. (*a*) 2 hr. previous exposure to 95% O_2 + 5% CO_2	
No tyramine added	770·9 (1 hr.)
Tyramine present	449·5 (1 hr.)
Exp. (*b*) 2 hr. previous exposure to 95% N_2 + 5% CO_2	
No tyramine added	518·5 (45 min.)
Tyramine present	601·3 (45 min.)

of the rate of oxidation of succinate. It is evident from these experiments that tyramine *per se* is ineffective in inhibiting succinate oxidation by brain and that a product of oxidation of tyramine is the responsible agent. This is clearly an aldehyde as shown by the results given in Table 10 and is most probably *p*-hydroxyphenylacetaldehyde since this aldehyde has been isolated as a product of oxidation of tyramine by amine oxidase [Richter, 1937].

Effects of tyramine on succinate oxidation in presence of mixtures of muscle succinic dehydrogenase and liver amine oxidase

It is evident that if the conclusions which have been recorded are correct, an inhibition of succinate oxidation after the addition of tyramine should take place in the presence of a mixture of liver amine oxidase and muscle succinic dehydrogenase, similar to that which occurs in the presence of brain suspensions. The following experiments indicate that this phenomenon does in fact occur.

Liver amine oxidase was prepared by grinding guinea-pig or rat liver with sand for 20 min. and mixing with an equal volume of 0·066 *M* phosphate buffer *p*H 7·4. This was squeezed through muslin and dialysed overnight against 0·066 *M* phosphate buffer *p*H 7·4. The dialysed solution was centrifuged and the centrifugate made up to twice the initial volume. This preparation contained an active amine oxidase and possessed also a little succinic dehydrogenase activity.

Muscle succinic dehydrogenase was prepared by mincing guinea-pig or rat muscle (using the Latapie mincer) and washing three or four times with ten times its volume of water, squeezing the preparation through muslin after each wash. The washed finely divided muscle was finally made up to twice its initial volume with 0·02 *M* phosphate buffer *p*H 7·4. This preparation of succinic dehydrogenase is practically free from amine oxidase.

When sodium succinate solution is added to a mixture of amine oxidase and succinic dehydrogenase to which tyramine has been previously added and aerobic incubation for 15–30 min. at 37° carried out, it is found that the velocity of O_2 uptake due to succinate oxidation is much smaller than if tyramine had not been added to the system. A typical result is shown in Table 12 where

Table 12

O_2 uptakes (μl.) at 37° by a mixture of liver amine oxidase and muscle succinic dehydrogenase during the period of 30 min. following the addition of succinate (0·033 *M*) to the system containing tyramine. The systems were allowed to incubate aerobically at 37° for 30 min. before addition of the succinate.

Mixed enzymes No substrate	Mixed enzymes + tyramine (0·0019 *M*)	Mixed enzymes + succinate (0·033 *M*)	Mixed enzymes + tyramine (0·0019 *M*) + succinate (0·033 *M*) added after 30 min. incubation
7·5	26·9	308·7	75·9

a mixture of 0·7 ml. succinic dehydrogenase preparation and 0·5 ml. amine oxidase preparation in phosphate buffer (0·033 *M*; *p*H 7·4) was used. In the absence of amine oxidase succinate oxidation by muscle succinic dehydrogenase is but little impeded by the addition of tyramine. Thus in one experiment the O_2 uptake by a succinic dehydrogenase preparation in presence of sodium succinate (0·033 *M*) in 1 hr. was 144·5 μl. and this was reduced by previous aerobic exposure to tyramine (0·019 *M*) for 1 hr. to 115·5 μl. There is evidently a small reduction in velocity of succinate oxidation due to the tyramine but whether this is due to the presence of a small amount of amine oxidase in the muscle preparation or to the presence of the amine itself has not yet been made clear. It is sure however that this reduction is small compared with that effected by the addition of liver amine oxidase to the system.

The effect of adding benzedrine to a mixture of amine oxidase and succinic dehydrogenase

When benzedrine is added to the system amine oxidase, succinic dehydrogenase, tyramine and succinate, the fall in the rate of succinate oxidation is impeded and may be completely prevented. This is seen in the results recorded in Table 13, where a mixture of 0·5 ml. succinic dehydrogenase preparation

Table 13

O_2 uptakes (μl.) at 37° by a mixture of amine oxidase and succinic dehydrogenase during the period of 2 hr. following the addition of succinate to the systems indicated below. The systems were allowed to incubate aerobically at 37° before the addition of the succinate.

Mixed enzymes Succinate (0·033 *M*)	Mixed enzymes Tyramine (0·0019 *M*) +succinate (0·033 *M*)	Mixed enzymes Benzedrine (0·0014 *M*) +succinate (0·033 *M*)	Mixed enzymes Tyramine (0·0019 *M*) +benzedrine (0·0014 *M*) +succinate (0·033 *M*)
286·3	86·9	262·8	262·0

and 0·1 ml. amine oxidase preparation in *M*/30 phosphate buffer *p*H 7·4 was employed. The succinate was not added to the systems until the mixed enzyme preparation had been allowed to incubate in presence of tyramine or a mixture of tyramine and benzedrine for 1 hr. at 37°.

Thus the effect of benzedrine in neutralizing the inhibition of succinate oxidation effected by tyramine in presence of brain tissue can be seen equally well when a mixture of liver amine oxidase and muscle succinic dehydrogenase is substituted for the brain suspension.

Protection of succinic dehydrogenase by succinate from the effect of *p*-hydroxyphenylacetaldehyde

The addition of a mixture of succinate and tyramine to a minced brain suspension in phosphate buffer solution (*p*H 7·4) at 37° leads to a velocity of O_2 uptake which differs not appreciably from that obtained when no tyramine is added. It is only when the tissue has been in contact aerobically with tyramine for a period of 15 min. or over that the diminished velocity of succinate oxidation is observed. A typical result is shown in Table 14.

Table 14

O_2 uptakes (μl.) at 37° in 2 hr. by minced rat brain tissue in presence of succinate or of a mixture of succinate and tyramine. In one part of the experiment succinate was added at the same time as the tyramine; in the other succinate was added 1 hr. after the brain tissue had been in contact with the tyramine.

Succinate added at the same time as the tyramine		Succinate added 1 hr. after incubation with tyramine had commenced	
Succinate (0·033 *M*) alone	Tyramine (0·0014 *M*) +succinate (0·033 *M*)	Succinate (0·033 *M*) alone	Tyramine (0·0019 *M*) +succinate (0·033 *M*)
455·1	474·9	405·5	300·9

This phenomenon is also seen when a mixture of liver amine oxidase and muscle succinic dehydrogenase is used instead of a brain suspension. Results are quoted in Table 15. These show that the addition of succinate at the same

Table 15

O_2 uptakes (μl.) in 15 min. at 37° by a mixture of amine oxidase (0·5 ml.) and succinic dehydrogenase (0·7 ml.) in presence of succinate and of a mixture of tyramine and succinate. The succinate was added either at the commencement of the experiment (i.e. with the tyramine) or at intervals of 15 min. and 30 min. after aerobic incubation with the tyramine had commenced.

Mixed enzymes Succinate (0·033 *M*) added at commencement of the experiment	Mixed enzymes Tyramine (0·0019 *M*) + succinate (0·033 *M*) added at commencement of the experiment	Mixed enzymes Tyramine (0·0019 *M*) + succinate (0·033 *M*) added 15 min. after commencement of the experiment	Mixed enzymes Tyramine (0·0019 *M*) + succinate (0·033 *M*) added 30 min. after commencement of the experiment
166·8	171·9	113·1	41·0

time as tyramine to a mixture of the two enzymes leads to no change in the velocity of oxidation of the succinate. Previous aerobic incubation of the enzymes with tyramine leads to a greatly decreased rate of oxidation of succinate when this is subsequently added. The longer the period of previous incubation the smaller is the subsequent rate of succinate oxidation.

Since tyramine oxidation by the mixture of enzymes takes place in presence of succinate as shown by the development of the aldehyde (dinitrophenylhydrazine-alkali test) it is evident that succinate protects succinic dehydrogenase from the product of tyramine oxidation.

This protection of succinic dehydrogenase by succinate from the effect of a toxic agent is not a new observation. It was shown by Hopkins *et al.* [1938] that succinate protects its dehydrogenase from the inactivating influence of oxidized glutathione. These phenomena can indeed be regarded as special cases of a general rule relating to the protection of enzymes by their substrates (and molecules of allied molecular structure) from toxic agents (cf. Quastel [1931 : 1932] on the protection of fumarase and urease by their substrates from the influence of toxic dyestuffs, or Dixon & Keilin [1936] on the protection of xanthine oxidase by its substrate from the action of cyanide). The results of Hopkins *et al.* would indicate that a thiol group in the succinic enzyme is partly responsible for the attachment of its substrate. It is not unreasonable to suggest that the toxic action of *p*-hydroxyphenylacetaldehyde might also be due to a condensation with a thiol group of the enzyme. It is evident however that if this is the case there is not the same reactivity with all aldehydes, for as has already been stated the inhibition of succinate oxidation in presence of brain takes place only with tyramine among the amines investigated.

Competition of amines for amine oxidase

The evidence submitted above shows the great importance of amine oxidase in brain in accounting for the stimulation of O_2 uptake by brain tissue effected by benzedrine when respiration takes place in presence of tyramine, etc. A knowledge of the nature of the competition between benzedrine and other amines for amine oxidase will be of value in assessing the relative importance of various amines as inhibitors of amine oxidase and in giving some conception of the concentrations at which various amines may be likely to stimulate brain respiration.

The fact that amines combine with amine oxidase in a strictly reversible manner may be shown by work which has been carried out on the competition between benzedrine and tyramine for amine oxidase (rat liver extracts).

Let us assume that the following reversible reactions take place:

$$E+T=ET,$$
$$E+B=EB,$$

where E = amine oxidase, T = tyramine, B = benzedrine or benzedrine derivative. Let x and y be the molar concentrations of tyramine and benzedrine respectively, and K_m and K_y the dissociation constants of ET and EB respectively. Let v = velocity of O_2 uptake effected by the amine oxidase in presence of tyramine alone and v' the velocity of O_2 uptake by the same amount of enzyme in presence of a mixture of tyramine and benzedrine.

Then, according to the mass action law,

$$\frac{v'}{v}=\frac{1}{1+\frac{K_m y}{K_y(x+K_m)}}.$$

If I = fractional inhibition of the activity of the enzyme effected by the benzedrine, then

$$I=\frac{v-v'}{v}$$

and

$$\frac{1-I}{I}=\frac{1}{\frac{v}{v'}-1}=\frac{K_y(x+K_m)}{K_m y}. \qquad \ldots(1)$$

That this expression is followed may be seen from Table 16 where velocities of O_2 uptake due to tyramine and to mixtures of tyramine and benzedrine in presence of amine oxidase (rat liver extract) are noted. Some of these results (Exp. 1, 2) were obtained by Pugh & Quastel (unpublished) using different quantities of extracts. The velocities of O_2 uptake with given quantities of tyramine, or tyramine and benzedrine, are constant over the initial periods of the experiments and the velocities recorded refer only to such initial periods. The results indicate that benzedrine competes with tyramine for amine oxidase in a strictly reversible manner. Taking $K_m=0{\cdot}00178$ [Blaschko *et al.* 1937, 1] and K_y = dissociation constant of benzedrine-amine oxidase complex = 0·0004 approximately, the calculated inhibitions of O_2 uptake due to the presence of benzedrine are approximately equal to the observed inhibitions (see Table 16).

Table 16

Inhibitions effected by benzedrine on O_2 uptake by amine oxidase preparations of rat and guinea-pig liver in presence of tyramine.

I (calc.) is given by equation (1). $K_m=0{\cdot}00178$; $K_y=0{\cdot}0004$.

Exp.	M conc. tyramine	M conc. benzedrine	Rate of O_2 uptake in absence of benzedrine μl. O_2 per 30 min.	Rate of O_2 uptake in presence of benzedrine μl. O_2 per 30 min.	I fractional inhibition of O_2 uptake due to benzedrine	I calc.
1 (rat)	0·0066	0·0033	180	57	0·68	0·65
2 (rat)	0·0066	0·0066	114	26	0·77	0·78
3* (guinea-pig)	0·01	0·00125	160·6	105·9	0·34	0·32
	0·01	0·0025	160·6	79·6	0·50	0·49
	0·01	0·0075	160·6	45·9	0·71	0·74

* The O_2 uptakes recorded in this experiment refer to a period of 45 min.

This indicates that the affinity of benzedrine for amine oxidase is over four times that of tyramine for the enzyme.[1]

l-*Ephedrine and tyramine*

It may be shown in a similar manner that *l*-ephedrine combines reversibly with amine oxidase. There is fair agreement between calculated and observed inhibitions of the O_2 uptakes when the dissociation constant of the ephedrine-amine oxidase complex is taken as 0·0057. On substituting this value together with $K_m = 0{\cdot}00178$ in equation (1), the results given in Table 17 are obtained.

Table 17

Inhibitions effected by *l*-ephedrine on O_2 uptakes by guinea-pig liver amine oxidase in presence of tyramine.

I (calc.) is given by equation (1). $K_m = 0{\cdot}00178$; $K_y = 0{\cdot}0057$.

M conc. tyramine	*M* conc. ephedrine	Rate of O_2 uptake in absence of ephedrine. μl. O_2 in 30 min.	Rate of O_2 uptake in presence of ephedrine. μl. O_2 in 30 min.	*I* fractional inhibition of O_2 uptake due to ephedrine	*I* calc.
0·01	0·025	75·2	44·7	0·40	0·40
0·01	0·05	75·2	31·0	0·59	0·57
0·01	0·075	75·2	25·7	0·66	0·66
0·01	0·1	75·2	22·9	0·70	0·73
0·01	0·125	75·2	16·3	0·78	0·77

*3:4-Methylenedioxyphenyliso*propylamine *and tyramine*

3:4-Methylenedioxyphenyl*iso*propylamine is a powerful inhibitor of amine oxidase, its effect being at least equal to that of benzedrine in this respect. Results illustrating this fact are shown in Table 18, where the dissociation constant for the 3:4-methylenedioxybenzedrine-amine oxidase complex is taken as 0·0004, that for tyramine-amine oxidase being 0·00178.

Table 18

M conc. tyramine	*M* conc. 3:4-methylenedioxy-phenyl*iso*propylamine	*I* (obs.) fractional inhibition of O_2 uptake (guinea-pig liver amine oxidase)	*I* (calc.)
0·01	0·00125	0·32	0·32
0·01	0·0025	0·48	0·49
0·01	0·005	0·61	0·65

*3-Methoxy-4-hydroxyphenyliso*propylamine *and tyramine*

The benzedrine derivative, 3-methoxy-4-hydroxyphenyl*iso*propylamine, also combines reversibly with amine oxidase. Taking the dissociation constant of this amine with amine oxidase to be 0·005 there is good agreement between the observed and calculated inhibitions of O_2 uptake due to rat liver amine oxidase in presence of tyramine. Results are shown in Table 19.

It will be observed that the affinity of 3-methoxy-4-hydroxybenzedrine for amine oxidase is a little more than that of *l*-ephedrine and is much less than that of benzedrine.

[1] The assumption is made in these calculations that the velocity of O_2 uptake during the initial period of the experiment is due mainly to the oxidation of tyramine to the corresponding aldehyde. Investigations are now being carried out to determine how far the apparent equilibria constants of amines with amine oxidase are affected by secondary oxidations in the presence of the enzyme.

Table 19

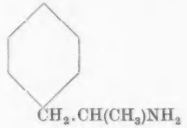
$K_m = 0{\cdot}00178$; $K_y = 0{\cdot}0050$.

M conc. tyramine	M conc. 3-methoxy:4-hydroxybenzedrine	I (obs.) fractional inhibition of amine oxidase	I (calc.)
0·01	0·0135	0·29	0·29
0·01	0·0217	0·38	0·39
0·01	0·0352	0·51	0·51

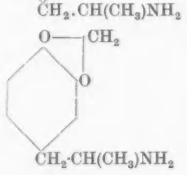
The following[1] is a table of the approximate dissociation constants of amine oxidase complexes of *l*-ephedrine, benzedrine and of benzedrine derivatives, taking that of the tyramine complex to be 0·00178:

Benzedrine	0·0004
3:4-Methylenedioxybenzedrine	0·0004
Tyramine	0·00178
3-Methoxy-4-hydroxybenzedrine ...	0·0050
l-Ephedrine	0·0057

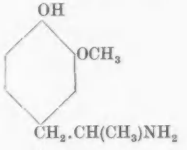
The structures of these molecules are given below:



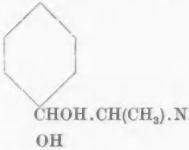
Benzedrine



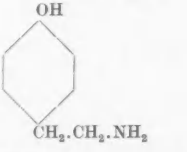
3:4-Methylenedioxyphenyl*iso*propylamine



3-Methoxy-4-hydroxyphenyl*iso*propylamine



Ephedrine



Tyramine

[1] The magnitudes of these constants are subject to revision in view of the present uncertainty of the K_m of the tyramine complex. This is given, for example, as 0·0005 by Kohn [1937]. The relative values, however, will remain approximately those shown in the table.

It would be expected from these results that *l*-ephedrine and 3-methoxy-4-hydroxybenzedrine would be less effective than benzedrine in neutralizing the inhibitory effects of tyramine oxidation on brain respiration in presence of glucose or succinate and that 3:4-methylenedioxyphenyl*iso*propylamine would be at least equal to benzedrine in this respect. These predictions may be verified as shown below.

Effects of ephedrine and 3:4-methylenedioxy*iso*propylamine on tyramine inhibitions of brain oxidations

As shown in Table 20 *l*-ephedrine is much inferior to benzedrine in retarding the inhibition of succinic acid oxidation due to the exposure of brain tissue to tyramine. The stimulant action of ephedrine on such an oxidation after exposure of brain tissue to tyramine ($0{\cdot}0019\,M$) may be quite plainly observed with

Table 20

O_2 uptakes (μl.) in 1 hr. at 37° by minced rat brain in presence of succinate, tyramine etc., as shown below. Succinate was added 2 hr. after the brain tissue had been in contact at 37° with the tyramine and with the mixtures of tyramine and ephedrine, and tyramine and benzedrine. Succinate = $0{\cdot}013\,M$.

Succinate	Succinate + tyramine ($0{\cdot}0019\,M$)	Succinate + ephedrine ($0{\cdot}00083\,M$) + tyramine ($0{\cdot}0019\,M$)	Succinate + benzedrine ($0{\cdot}0004\,M$) + tyramine ($0{\cdot}0019\,M$)
215·1	112·2	124·9	174·1

relatively high concentrations of *l*-ephedrine, e.g. $0{\cdot}004\,M$. For example under these conditions an O_2 uptake by brain tissue in presence of succinate ($0{\cdot}013\,M$) and tyramine of 123 μl. was increased to 191·6 μl. by the addition of *l*-ephedrine ($0{\cdot}0041\,M$). 3:4-Methylenedioxyphenyl*iso*propylamine was equal in activity to benzedrine in neutralizing the inhibiting influence of tyramine oxidation on succinate breakdown. Its powerful activity can also be seen in its retarding action on the fall of respiration of brain tissue in presence of glucose when tyramine is present in the system. A typical result is given in Table 21.

Table 21

Conditions as in Table 3. Glucose = $0{\cdot}01\,M$. Tyramine = $0{\cdot}0019\,M$. Air. 37°.

	μl. O_2 uptake		
	1st hour	2nd hour	3rd hour
Glucose	276·1	209·3	182·3
Glucose + tyramine	209·9	98·4	37·1
Glucose + tyramine + 3:4-methylenedioxyphenyl*iso*propylamine ($7{\cdot}7 \times 10^{-5}\,M$)	215·7	112·9	50·7
Glucose + tyramine + 3:4-methylenedioxyphenyl*iso*propylamine ($1{\cdot}5 \times 10^{-4}\,M$)	211·9	122·7	68·7
Glucose + tyramine + 3:4-methylenedioxyphenyl*iso*propylamine ($7{\cdot}7 \times 10^{-4}\,M$)	178·5	117·5	93·5

A comparison of the effects of benzedrine, *l*-ephedrine and 3-methoxy-4-hydroxybenzedrine in retarding the inhibitory action of tyramine on the respiration of brain cortex slices in presence of glucose is given in Table 22. It will be noted that both *l*-ephedrine and 3-methoxy-4-hydroxybenzedrine are less effective than benzedrine in effecting an increased rate of O_2 uptake.

Table 22

Rates of O_2 uptake by rat brain cortex slices in glucose (0·01 *M*)-phosphate (0·03 *M*; *p*H 7·4)-Locke media in the presence of tyramine and mixtures of tyramine and benzedrine, *l*-ephedrine or 3-methoxy-4-hydroxybenzedrine.

Amines present	Q_{O_2} 1st hour	2nd hour	3rd hour
Tyramine ($1·9 \times 10^{-3}$ *M*)	12·3	7·7	4·6
Tyramine ($1·9 \times 10^{-3}$ *M*) + benzedrine ($4·3 \times 10^{-4}$ *M*)	12·5	11·0	9·9
Tyramine ($1·9 \times 10^{-3}$ *M*) + ephedrine ($1·5 \times 10^{-3}$ *M*)	12·0	9·6	7·0
Tyramine ($1·9 \times 10^{-3}$ *M*) + 3-methoxy-4-hydroxybenzedrine ($1·4 \times 10^{-3}$ *M*)	12·1	10·4	8·3

The action of benzedrine on the inhibition of brain respiration caused by luminal, chloretone and bulbocapnine

Benzedrine has apparently no neutralizing action, at the concentrations investigated, on the inhibiting influence of the narcotics luminal (phenylethylbarbituric acid) and chloretone, and of bulbocapnine on the respiration of brain tissue in presence of glucose. This lack of activity on the part of benzedrine would be expected if its influence is mainly related to its affinity for amine oxidase. Illustrative results are shown in Table 23.

Table 23

O_2 uptakes by minced rat brain tissue in glucose (0·01 *M*)—phosphate-saline at 37° in the presence of luminal, chloretone and bulbocapnine with and without benzedrine.

	Substrates	μl. O_2 1st hour	2nd hour	3rd hour
Exp. 1	Glucose	307·9	248·5	209·6
	Glucose + luminal (0·08 %)	155·7	104·9	83·3
	Glucose + luminal + benzedrine ($7·1 \times 10^{-4}$ *M*)	132·4	90·8	78·2
Exp. 2	Glucose	279·1	223·5	187·5
	Glucose + chloretone (0·033 %)	163·8	131·6	111·1
	Glucose + chloretone + benzedrine ($5·7 \times 10^{-4}$ *M*)	144·4	107·8	97·9
Exp. 3	Glucose	288·1	239·5	217·1
	Glucose + bulbocapnine (0·01 %)	159·3	146·6	139·3
	Glucose + bulbocapnine + benzedrine ($4·3 \times 10^{-4}$ *M*)	171·5	154·1	143·0

Possible clinical significance of the action of benzedrine on brain metabolism

It is now well known that benzedrine administration has very considerable value in cases of narcolepsy and in conditions where a stimulation of the central nervous system is required. It was indeed the clinical value of benzedrine administration which led us to the investigation which has just been described.

The effects of benzedrine *in vitro* in partially neutralizing the inhibition of glucose oxidation by brain due to the presence of tyramine and other amines take place at concentrations which are not markedly greater than those which have pharmacological effects *in vivo*. Moreover, since it has been shown that competition between benzedrine and other amines takes place at the amine

oxidase according to the laws of mass action it follows that the influence of benzedrine may be exerted in the body at much lower concentrations than those which we have investigated. It seems not unreasonable to suggest that the action of benzedrine *in vivo* is linked with its ability to compete with amines giving rise in the body to toxic substances; the lower the concentration of such amines the lower the quantity of benzedrine which will be required to compete successfully with them.

Ephedrine is known clinically to be less powerful than benzedrine as a stimulant of the central nervous system and it has been shown that such a difference between their effects on brain respiration occurs also *in vitro*. According to Gunn *et al.* [1939] 3:4-methylenedioxyphenyl*iso*propylamine is a somewhat more powerful stimulant of the central nervous system than benzedrine, and it has just been seen that the former amine has at least the same activity as benzedrine in neutralizing the fall of brain respiration in a glucose medium due to tyramine.

Again, according to Gunn *et al.* [1939], 3-methoxy-4-hydroxyphenyl*iso*-propylamine is a less powerful stimulant of the nervous system than benzedrine. This result is consistent with the relatively poor activity of the former amine in neutralizing the inhibitory action of tyramine on brain oxidations. Such results are suggestive of a possible connexion between amine metabolism in the brain and the development of the clinical condition (fatigue) known to be relieved by benzedrine administration.

It will be of much interest to investigate cases of narcolepsy with a view to observing whether aldehydes, or similar toxic bodies, are elaborated in this disorder, and if so, whether their rates of formation are retarded by benzedrine administration.

Summary

1. When brain respiration in a glucose medium takes place in the presence of tyramine, β-indolethylamine or *iso*amylamine a fall in respiration occurs. This fall in respiration is neutralized by the presence in the system of benzedrine (β-phenyl*iso*propylamine sulphate). This phenomenon applies to the respiration of intact brain cortex slices or of minced brain tissue.

2. The same phenomenon obtains in the case of tyramine when glucose is replaced by sodium succinate.

3. The fall in brain respiration due to the presence of tyramine, etc., is not due wholly to the amine itself but to a product of oxidation of the amine, i.e. the corresponding aldehyde, or a product formed by further metabolism of the aldehyde.

4. Succinate protects succinic dehydrogenase from the toxic influence of the oxidation product of tyramine (probably *p*-hydroxyphenylacetaldehyde).

5. Benzedrine owes its stimulating influence to its ability to compete reversibly with amines for the amine oxidase of brain and of other organs, thereby reducing the rate of formation of inhibitory aldehyde.

6. *l*-Ephedrine, 3:4-methylenedioxyphenyl*iso*propylamine and 3-methoxy-4-hydroxyphenyl*iso*propylamine also compete reversibly with tyramine for amine oxidase and they all show effects similar to benzedrine in their retarding actions on the fall of respiration of brain tissue in glucose or succinate media due to the presence of tyramine. Ephedrine and 3-methoxy-4-hydroxyphenyl-*iso*propylamine are much less active than benzedrine, and 3:4-methylene-dioxyphenyl*iso*propylamine has about the same activity as benzedrine. The equilibrium constants of these amines with amine oxidase have been determined.

7. Aldehydes such as *iso*valeric aldehyde, and *p*-hydroxybenzaldehyde are inhibitory to the respiratory processes of brain and their inhibitory effects are not neutralized by the addition of benzedrine.

8. Benzedrine has no retarding action on the fall of respiration of brain tissue in a glucose medium due to the presence of luminal, chloretone or bulbocapnine.

9. The possible clinical significance of these results is discussed. It is pointed out that the relative stimulating actions of ephedrine, benzedrine and benzedrine derivatives on brain respiration in the presence of tyramine bear a parallelism to the relative stimulating actions of these substances on the central nervous system as observed *in vivo*.

Our grateful thanks are due to the Medical Research Council for a grant in aid of the equipment of this laboratory, to the Rockefeller Foundation for a maintenance grant to one of us and to the Ella Sachs Plotz Foundation for a grant for the purchase of animals required for this work. We are also grateful to Messrs Menley and James, Ltd., for a gift of benzedrine sulphate and to Prof. Gunn for samples of 3:4-methylenedioxyphenyl*iso*propylamine and 3-methoxy-4-hydroxyphenyl*iso*propylamine.

REFERENCES

Blaschko, Richter & Schlossman (1937, 1). *Biochem. J.* **31**, 2187.
—— —— —— (1937, 2). *J. Physiol.* **90**, 1.
Dixon & Keilin (1936). *Proc. roy. Soc.* B, **119**, 159.
Gunn, Gurd & Sachs (1939). *J. Physiol.* **95**, 485.
Harrison (1932). *Biochem. J.* **26**, 472.
Hopkins, Morgan & Lutwak-Mann (1938). *Biochem. J.* **32**, 1829.
Kohn (1937). *Biochem. J.* **31**, 1693.
Philpot (1937). *Biochem. J.* **31**, 856.
Pugh & Quastel (1937, 1). *Biochem. J.* **31**, 286.
—— —— (1937, 2). *Biochem. J.* **31**, 2306.
Quastel (1931). *Biochem. J.* **25**, 898.
—— (1932). *Biochem. J.* **26**, 1685.
—— & Wheatley (1933). *Biochem. J.* **27**, 1609.
—— —— (1938). *Biochem. J.* **32**, 936.
Richter (1937). *Biochem. J.* **31**, 2022.

54. PROLIFERATION-PROMOTING INTERCELLULAR HORMONES

I. QUANTITATIVE STUDIES OF FACTORS PRODUCED BY INJURED ANIMAL TISSUE CELLS

By JOHN R. LOOFBOUROW, SISTER CECELIA MARIE DWYER, S.C., and SISTER MARY MICHAELLA LANE, S.C.

From the Research Laboratories of Institutum Divi Thomae, Cincinnati, Ohio, and the Research Laboratory of Good Samaritan Hospital, Dayton, Ohio

(*Received 8 January 1940*)

In investigating factors from cells which influence cellular proliferation [Fardon *et al.* 1937; Sperti, Loofbourow & Dwyer, 1937, 1, 2], respiration [Norris & Kreke, 1937; Cook *et al.* 1938], and glycolysis [Schroeder & Cook, 1939] our studies of proliferation-promoting factors from injured animal tissue cells have been carried out with tissue cultures as a means of assaying the products obtained [Sperti, Loofbourow & Lane, 1937; Loofbourow, Cueto & Lane, 1939]. In the case of similar proliferation-promoting factors from injured yeast cells, the assays on yeast have afforded much more accurate quantitative data [Loofbourow, Dwyer & Morgan, 1938]. Since it is desirable to obtain a comparable degree of accuracy in the assay of animal-cell products in order to answer various questions regarding the physiological production and chemical nature of the factors, we endeavoured to see if the animal-cell products could be assayed on yeast. The yeast assay technique having proved to be practicable, we wish to present here the results of quantitative studies by this method of the products from injured animal tissue cells.

The name "intercellular hormones" [Loofbourow & Morgan, 1938] has been suggested for factors controlling cellular metabolism which are released by cells and act from cell to cell through the intermediary of the intercellular fluids. Thus the respiratory, glycolytic and proliferation-controlling factors investigated in our laboratories, as well as Törö's [1937, etc.] corhormone, Wildiers' [1901] bios, Williams' [1933, etc.] pantothenic acid, Bonner's [1937, etc.] traumatin, the auxins and many other such substances are probably all "intercellular hormones" in this sense. The particular proliferation-promoting intercellular hormone discussed in what follows appears to be produced especially by injured cells, and is thus a "wound hormone" in the sense used by Wiesner [1892], Haberlandt [1921], Reiche [1924] and others for growth factors obtained from injured plant tissues. For this reason, we have at times called it an "intercellular wound hormone", but it should be pointed out that the same proliferation-promoting factor appears to occur in the intercellular fluids from uninjured cells, only to a less extent, and the relation to injury is therefore evidently a quantitative rather than a qualitative one.

Experimental

As a means of injuring the tissue cells, lethal ultraviolet from a 500 w. Burdick quartz mercury arc, operated at 65 v. across the arc, was used in all of the following experiments. The irradiation distance was uniformly 25 cm.

Irradiation was carried out in quartz test tubes, with constant stirring. The control and irradiated tissues were always kept at the same temperature. Lethal ultraviolet is a convenient means of producing cellular injury because its effects can easily be controlled.

Three sources of tissue were used: adult newts, 7-day chick embryos and 14-day rat embryos.

In the newt experiments, whole newts were washed and minced in Ringer's solution. The suspensions of minced tissues were divided into two portions, one of which was irradiated for 1–1·25 hr. Following irradiation, both suspensions were centrifuged, and the supernatant fluids were decanted and taken to dryness in an oven.

The chick embryos were removed under sterile conditions, and minced in Drew's solution. Irradiation, centrifuging and drying followed the technique described above.

The rat embryos were removed under sterile conditions from 1-year-old pregnant females. They were minced in Drew's solution or 0·89 % NaCl and washed three or more times in changes of the solution to remove growth factors arising from mechanical injury [Fischer, 1930; Fardon *et al.* 1938; 1939; Loofbourow, Cueto & Lane, 1939]. The tissues were divided into two approximately equal portions, each of which was suspended in 10 ml. of the solution. Irradiation, centrifuging and drying were carried out as above. Special precautions were taken in these experiments to keep the tissues at or near 37° throughout all manipulations.

The assays of the animal tissue products on yeast were made by adding serial dilutions of the preparations to cultures of *S. cerevisiae* grown in Reader's medium [Reader, 1927] in rocker tubes [Norris & Kreke, 1937] for 24 hr. at 30°. The concentration of yeast used for seeding and the crop after 24 hr. were determined photoelectrically in terms of the concentration of yeast protoplasm in mg. per ml. [Loofbourow & Dwyer, 1938, 2]. Haemacytometer counts showed that the increase in protoplasm measured in these experiments is accounted for by increase in cell number rather than by increase in cell size. Consequently, such products as were found to be growth-promoting in the assays here reported were proliferation-promoting factors.

Each tube contained 24 ml. of Reader's medium and 1 ml. of added material (0·89 % NaCl in the case of controls). The yeast used for seeding was washed with Reader's medium from 48 hr. slants on Saboraud's medium and added to the rocker tubes in such dilution as to seed them to a concentration of 0·064 mg. per ml.

When the yield of yeast after 24 hr. was plotted against the concentration of test material added, a linear relationship usually resulted within the range of values used (Fig. 1) as in the case of yeast products [Loofbourow, Dwyer & Morgan, 1938], though for some preparations the relationship was non-linear (Fig. 2), as in the case of certain preparations we have obtained from bacteria [Loofbourow & Morgan, 1939].

As a standard unit of growth, a crop of 1·6 mg. of yeast per ml. greater than the control crop was used, as in previous work [Loofbourow, Dwyer & Morgan, 1938]. The quantity (mg.) of tested material in each ml. of rocker tube contents required to give such a crop was determined from the curves. Thus there was determined the number of mg. of tested material per "growth unit" (the reciprocal of which gave the number of growth units per mg.) in terms of a standard, reproducible biological effect.

In comparing the potencies of materials which give different shapes of growth-concentration curves, it is necessary to beware of false interpretations because

of the fact that the apparent potency ratios of such materials are not the same at different concentration levels. We have not found, as yet, a simple method of avoiding this difficulty, and hence have limited ourselves to pointing out the salient facts about those particular instances in which there was a marked departure from a linear relationship in the concentration ranges employed.

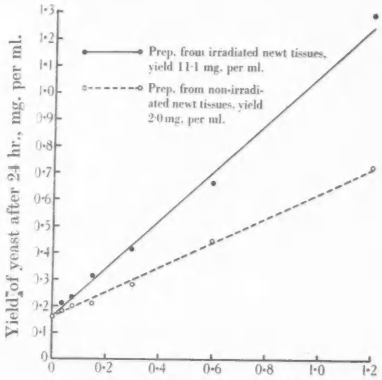

Prep. from irradiated newt tissues, yield 11·1 mg. per ml.
Prep. from non-irradiated newt tissues, yield 2·0 mg. per ml.
Yield of yeast after 24 hr., mg. per ml.
Concentration in rocker tubes, mg. per ml.

Fig. 1.

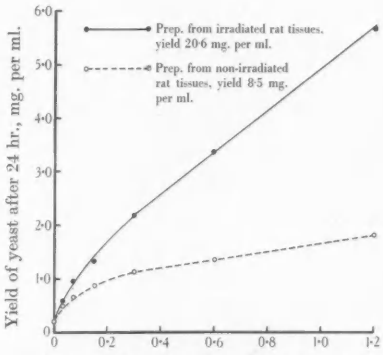

Prep. from irradiated rat tissues, yield 20·6 mg. per ml.
Prep. from non-irradiated rat tissues, yield 8·5 mg. per ml.
Yield of yeast after 24 hr., mg. per ml.
Concentration in rocker tubes, mg. per ml.

Fig. 2.

Fig. 1. Typical assay of intercellular wound hormone from irradiated newt tissues. It should be noted that not only was the potency of equal weight of material from the irradiated tissues approximately twice that from non-irradiated tissues, but the yield of material from the irradiated tissues was also about 5·5 times as great. Thus, equal volumes of the two filtrates showed growth potency ratios of about 11 to 1.

Fig. 2. Assay of intercellular wound hormone from irradiated rat tissues selected to illustrate the non-linear growth concentration relationship obtained with some preparations. Note increased yield of material from irradiated tissues as well as increased potency per unit weight.

Table 1 summarizes several typical assays of preparations from newts, chick embryos, rat embryos and yeast, selected to illustrate various points.

In all the animal preparations tested, the intercellular fluids from irradiated tissues showed greater growth potencies than those from corresponding non-irradiated tissues. If the ratio of potencies of test and control filtrates (Table 1, column H) of animal tissues is compared with that of the representative yeast preparation listed, it is seen, however, that the ratio for the yeast preparation is roughly nine times as great as the highest ratio for the animal preparations. Reference to columns D, E and F indicates that this is due to the greater potencies and yields of control filtrates in the case of the animal tissue products. We believe this to be explained by the greater production of proliferation-promoting factors in the control animal tissues by mechanical injury, and the greater release in these tissues of dead-cell disintegration products.

The results demonstrate the necessity of the utmost care in washing animal tissues to free them from such growth factors when they are to be used in tissue cultures for growth tests or in the study of the effects of various agents in causing them to produce growth factors [compare Fardon & Sullivan, 1938; Fischer, 1930].

Autoclaving for 20 min. at 15 lb. did not destroy the active factor, as shown by preparation No. 3·7 from newts. Filtration through Berkefeld "N" filters

Table 1. *Results of typical assays of intercellular wound hormone preparations from animal tissues*

A	B	C	D	E	F	G	H	I
Preparation	Irradiation time	Concentration of tissue	Yield mg. per ml.	Growth units per ml.	Growth units per ml.	Growth units per g. tissue	Ratio irrad- to non-irrad. g.u. per ml.	Extinction at 2600 Å.
3·25 from newts	1 hr. 15 min.	—	11·0 n.	0·57 n.	6·3	—	10·5	—
	Non-irradiated	—	2·0 n.	0·29 n.	0·6	—		—
3·7 from newts, autoclaved 20 min. at 15 lb.	1 hr.	—	—	—	6·0	—	—	—
3·29 from chick embryos, unwashed	25 min.	—	—	—	27·2	—	1·3	—
	Non-irradiated	—	—	—	21·8	—		—
11·11 from rat embryos	2 hr. 55 min.	2·24 g. to 1 ml. Drew	41·3	8·9	367·5	164	2·1	2·92
	Non-irradiated	2·66 g. to 1 ml. Drew	29·8	5·8	172·7	65		1·78
11·161 from rat embryos	3 hr.	0·93 g. to 1 ml. saline	25·8	9·6	250·3	272	4·9	—
	Non-irradiated	1·33 g. to 1 ml. saline	13·5	3·8	51·2	39		—
11·162 from rat embryos (11·161 after Berkefeld filtration)	See preparation 11·161		23·5	9·5	223·4	240	5·7	3·1
			10·3	3·8	39·2	29·5		1·29
11·252 from rat embryos, Berkefeld filtrate	4 hr.	1·72 g. to 1 ml. saline	18·0	6·9	124·2	72	8·7	2·50
	Non-irradiated	1·89 g. to 1 ml. saline	6·2	2·3	14·3	7·6		0·89
11·2 from rat embryos, Berkefeld filtrate	61 min.	1·77 g. to 1 ml. Drew	20·4	5·8	118·4	67	2·7	1·84
	Non-irradiated	1·84 g. to 1 ml. Drew	23·0	1·9	43·6	23·7		1·16
4·26 from yeast, Berkefeld filtrate	7 hr.	0·1 g. per ml. saline	14·3 n.	5·0 n.	71·5	715	87·2	—
	Non-irradiated		0·59 n.	1·39 n.	0·82	8·2		—

Note. The weights used in calculating yields and potencies were the total weights of the dried filtrates, including the salts, etc., from the solutions in which the cells were suspended, except in those instances marked "n." (for "net"), in which allowance was made for the weight of such materials.

did not remove the factor, as illustrated by comparison of rat preparations No. 11·161 and No. 11·162. These are typical of results obtained repeatedly. If we may presume that the factor active on animal tissues is identical with that active on yeast, we must conclude that our preliminary indications [Loofbourow, Cueto & Lane, 1939] that Berkefeld filtration removes the active factor were in error.

The ultraviolet absorption spectra of the preparations had a maximum near 2600 Å. (Fig. 3) characteristic of purines and pyrimidines of the nucleic acid complex [Heyroth & Loofbourow, 1931; 1934], as in the case of preparations from yeast. On the basis of spectrographic and chemical studies of the yeast preparations [Cook, Loofbourow & Stimson, 1938; Loofbourow, Cook & Stimson, 1938], it has been suggested that the active yeast product contains adenine, guanine, pentose and phosphorus, and is free from pyridine, protein and sulphur. While the spectrographic curves of the tissue products are broader than those of the yeast products, their common maximum near 2600 Å. and the rather close correlation between the extinctions of the animal products at this wave-

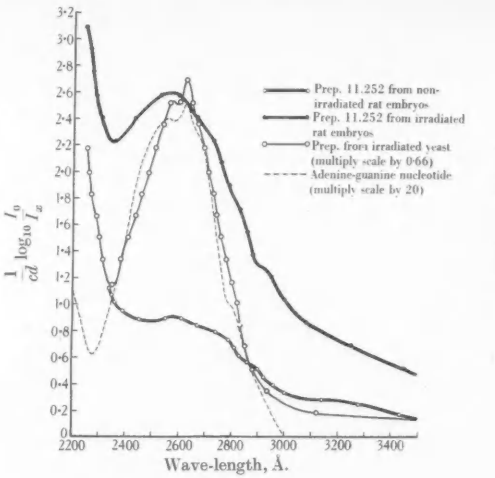

Prep. 11.252 from non-irradiated rat embryos
Prep. 11.252 from irradiated rat embryos
Prep. from irradiated yeast (multiply scale by 0·66)
Adenine-guanine nucleotide (multiply scale by 20)
$\frac{1}{cd}\log_{10}\frac{I_0}{I_x}$
Wave-length, Å.

Fig. 3.

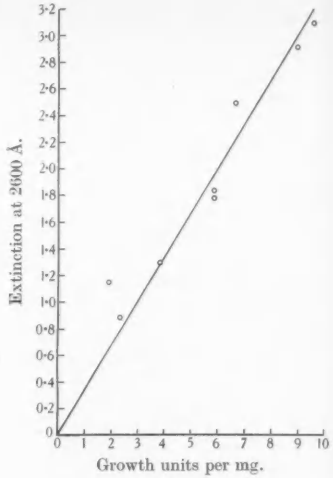


Fig. 4.

Fig. 3. Ultraviolet absorption spectra of typical preparations from rat tissues and yeast. The spectra of the yeast preparation and of the hypothetical guanine-nucleotide-adenine-nucleotide complex are plotted to different extinction scales to facilitate comparison.

Fig. 4. Correlation of potency and ultraviolet absorption at 2600 Å. in various preparations.

Table 2. *Tests of the growth-promoting activities of amino-acids, nucleic acids etc. on yeast*

Material	*p*H adjusted to	Concentration range tested mg. per ml.	Growth units per mg.
Intercellular wound hormone prep. 11·16 from rat embryos	—	0·038–1·2	9·6
Arginine	7·3	0·038–1·2	0·06
Cystine	7·25	0·0038–0·12	Inactive
d-Alanine	7·4	0·096–0·31	Slight activity in low concentrations (see Fig. 5)
l-Alanine	7·2	0·038–1·2	Inactive
dl-Alanine	7·3	0·037–1·18	? Slight activity
β-Alanine	7·4	0·041–1·30	Inactive
Tryptophan	7·3	0·037–1·19	Inactive
Tyrosine	7·4	0·045–1·45	Inactive
d-Glutamic acid	7·3	0·027–0·86	Inactive
Histidine	7·22	0·034–1·1	Inactive
dl-Phenylalanine	7·2	0·019–0·06	Inactive
Glycine	7·4	0·045–1·45	Inactive
l-Leucine	7·36	0·0092–0·29	Inactive
l-Aspartic acid	7·39	0·028–0·88	Slight activity in low concentrations (see Fig. 5)
l-Proline	7·39	0·025–0·8	Inactive
Inositol	7·2	0·033–1·08	Inactive
Yeast nucleic acid	7·25	0·036–1·16	0·02
Thymus nucleic acid	7·39	0·038–1·2	Inactive
Adenosine	—	0·038–1·2	0·03
Aneurin	7·3	0·038–1·2	Inactive
Riboflavin	—	0·038–1·2	Inactive
Nicotinic acid	7·25	0·038–1·2	Inactive
Insulin (8 units per mg.)	—	0·063–0·2	Inactive

length and their potencies (Fig. 4) suggests that the active factors are similar in the yeast and animal preparations.

Since it was possible that the difference in the ultraviolet absorptions of the animal and yeast products might be due to the presence in the former of greater quantities of amino-acid residues from proteins, we undertook a study of the activity of various amino-acids in yeast growth experiments, and at the same time tested certain other materials that might logically be suspected as contributing to the activity. The results are shown in Table 2.

The sensitivity of the method was sufficient to show a growth activity of about 0·01 growth unit per mg. All of the amino-acids tested had activities less than this, except arginine, *l*-aspartic acid and *d*-alanine. The two latter showed some growth activity in small concentrations, which rapidly fell off as the concentration increased. In neither of these could the activity be determined by extrapolation because of the non-linear characteristics of the growth-concentration relationships (Fig. 5). Comparison of their growth curves with those

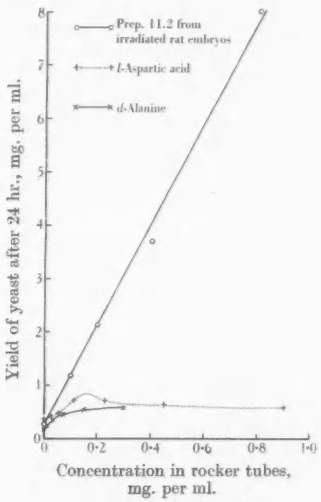


Fig. 5.

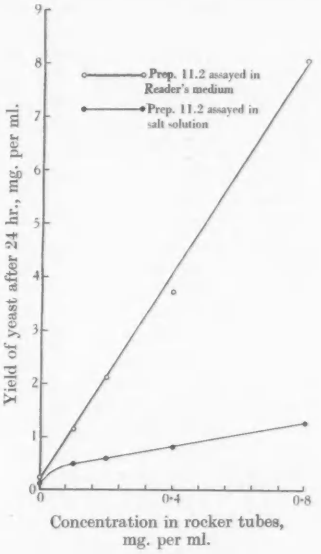


Fig. 6.

Fig. 5. Assays of *l*-aspartic acid and *d*-alanine compared with an assay of preparation 11·2 from rat tissues.

Fig. 6. Assays of rat tissue preparation 11·2 in Reader's medium and in salt solution.

of the animal preparation included on the figure show that they had insufficient effect to account for the activities of the animal preparations. The shapes of their growth-concentration curves suggest that such materials may account for the non-linear growth-concentration relationships at low concentrations in some of the wound hormone assays illustrated by the example shown in Fig. 2.

Arginine had an activity of 0·06 g.u. per mg.; *l*-alanine was inactive; *dl*-alanine showed a slight indication of activity, apparently attributable to

the *d*-alanine component. The only other materials with measurable activity were yeast nucleic acid (0·02 g.u. per mg.) and adenosine (0·03 g.u. per mg.). These values compare with 9·6 g.u. per mg. for the most potent rat preparation and 5·0 g.u. per mg. for the yeast preparation of Table 1.

It is evident that none of the materials tested can account for the activity of the proliferation-promoting intercellular hormone as determined in our growth experiments. This, of course, does not preclude the possibility of such substances acting as active growth factors for other strains of yeast, or for the same strain under other growth conditions, but that is a separate problem with which we were not concerned, our one purpose being to determine if the substances in question could account for the measured activity of the intercellular wound hormone preparations.

The question of whether the active material in the rat preparations acts directly as a nutrient or is effective in increasing the utilization of carbohydrate by yeast was investigated by comparing the growth of yeast in salt solution to which a rat preparation was added with its growth in Reader's medium (of which the nutrient component is dextrose) containing the same factor (Fig. 6). The 24 hr. yield of yeast in the controls was twice as great in Reader's medium as in salt, whereas in the rocker tubes containing 0·8 mg. per ml. of rat preparation No. 11·2, the yield was approximately seven times as great in Reader's medium as in salt. Thus, while there are apparently present in the rat preparations materials which support growth directly, the major part of the growth-promoting effect is evidently due to stimulation of carbohydrate metabolism.

Discussion

It should be emphasized that we do not regard ultraviolet irradiation as having any special significance in the production of the proliferation-promoting intercellular hormone other than that of a cell-injury agent. We have had comparable success in obtaining the factor by injuring cells mechanically [Loofbourow, Cook, Dwyer & Hart, 1939], chemically [Loofbourow & Dwyer, 1938, 1; 1939], by heat (unpublished investigations), by X-rays [Fardon *et al.* 1937], and by subjecting cells to anaerobiosis [Loofbourow & Dwyer, 1939]. Harker [1938] has shown that radium radiation leads to the release by yeast cells into the intercellular fluids of substances accelerating sucrose inversion. There is thus, so far as we know, no special effectiveness of ultraviolet radiation in causing cells to produce intercellular hormones. Ultraviolet irradiation of the cell-free filtrates from non-irradiated cells leads to no increase in proliferation-promoting activity.

The increased yield of the proliferation-promoting factor in the intercellular fluids when cells are injured is apparently due to the production of the factors by living cells as a physiological response to such conditions, and not due to dead-cell disintegration products [Loofbourow, Dwyer & Morgan, 1938; unpublished investigations of Loofbourow & Dwyer]. If the production of proliferation-promoting intercellular hormones by living cells is, as we believe, increased quite generally when cells are injured, these proliferation-promoting factors must be taken into account in considering such physiological processes as wound healing, overgrowth and neoplasia. The possible relationship of such substances to neoplasia has already been considered in a preliminary way [Sperti, Loofbourow & Dwyer, 1937, 2]. We have recently reported investigations in which the injection of the preparations from rat-embryo tissues described in this paper led to the production of local overgrowths, or tumours [Loofbourow, Cueto, Whalen & Lane, 1939].

In earlier publications [Loofbourow, Cook & Stimson, 1938; Cook, Loofbourow & Stimson, 1938] we pointed out that the shapes of the absorption curves of the active preparations are such as to suggest the presence of guanine as well as adenine. Recently Claude [1939], in an interesting study of the active factor of chicken tumour I, has found it to contain a lipin portion and a nucleoprotein portion, the latter being characterized by an absorption spectrum similar to the spectra of our preparations. From the position of the maximum of his spectrum near 2575 Å., and other evidence, he deduced that his nucleoprotein fraction contains guanine as a predominant part of the molecule. This, together with the fact that he has recently obtained similar fractions from chick and rat embryo tissues, and the fact that repeated injection of our proliferation-promoting intercellular hormone apparently leads to the production of tumours in rats, strongly suggests a similarity of the nucleic acid-like component of his preparations to our intercellular wound hormone.

In collaboration with Prof. E. S. Cook and his associates we have obtained preliminary evidence that our intercellular wound hormone stimulates yeast fermentation. That it is apparently produced in larger quantities under conditions of oxygen lack has already been mentioned. Schroeder & Cook [1939] have found that respiration-stimulating intercellular hormones obtained by them from normal cells and tissues lead to stimulation of the respiration and depression of the anaerobic and aerobic glycolysis of certain tumours *in vitro*, as well as of normal tissues. Davis [1937] has produced tumours in rats by ligating the arteries supplying one horn of the uterus, thus bringing about local oxygen want. Orr [1935] has shown that oxygen lack produced in the epithelium by the ligation of arteries or the injection of adrenaline leads to the more rapid production of tumours by dibenzanthracene applied to the affected areas. Cook *et al.* [1939] have demonstrated that dibenzanthracene depresses the respiration of yeast. In viewing these diverse findings in relation to each other, it seems possible that the metabolism of tissues generally may be controlled in large part by a suitable balance of intercellular hormones affecting proliferation, respiration and glycolysis, and that prolonged injury to appreciable volumes of cells, or prolonged oxygen lack, may lead, through the mechanism of an unbalanced production of such factors, to the abnormal proliferation and glycolysis characteristic of many types of tumour tissue.

The evidence thus far accumulated with regard to the chemical nature of our proliferation-promoting intercellular hormone points toward its being a guanine-adenine-nucleotide complex. On this assumption, a combined curve of the hypothetical absorption of guanine-nucleotide and adenine-nucleotide has been plotted for comparison of its shape (the magnitudes of absorption at different wave-lengths cannot of course be compared because the degree of purity of the intercellular hormone preparations is not known) with the shapes of the curves of the yeast and rat preparations (Fig. 6), using unpublished data of Stimson & Loofbourow for the absorption of adenine and guanine at the *p*H characteristic of the intercellular hormone solutions. The resemblance to the yeast preparation is close. Absorbing impurities may possibly account for the high minimum (2375 Å.) and high absorption in the long-wave region by the rat preparation and for the position of the minimum in the yeast preparation at about 2375 Å. The absorption of amino-acids might account for some of these effects. Both tyrosine and tryptophan have minima near 2400 Å. in acid solution [Holiday, 1936]. Other amino-acids with "end absorption" would produce the same effect of shifting the minimum toward longer wave-lengths. It is interesting, however, that, although chemical tests have thus far not indicated its presence

in our intercellular wound hormone preparations, nicotinic acid amide has a minimum at about 2400 Å., and the possibility that it may occur in our preparations cannot be excluded entirely until more highly purified samples have been shown to be free from it.

The extinction at 2600 Å. of the guanine-nucleotide adenine-nucleotide complex is roughly twenty times that of rat preparation No. 11·252. Consequently if the active material is of this nature, one would expect pure preparations to have an activity roughly twenty times that of rat preparation No. 11·252, or 138 g.u. per mg.

Spectrographic analysis would be greatly facilitated by the use of more highly purified preparations. Investigations soon to be published in collaboration with Sister Miriam Michael Stimson and others show that the active material can be obtained in more highly purified form by adsorption on magnesium oxide in Tswett columns. At least two adsorbed fractions are obtained, one fluorescing brilliantly yellow in ultraviolet light, the other fluorescing blue. Of these, the yellow-fluorescing fraction appears to be the more active.

The apparent relationship of the proliferation-promoting intercellular hormone to nucleic acid derivatives is of particular interest in view of Fischer's [1939] recent finding of a nucleoprotein growth factor for tissue cultures in beef embryo extracts, and of Caspersson's [1936; 1939] ultraviolet microscope studies showing increase in the nucleic acid content of cells during mitosis. It should be noted that the factor which Fischer isolated is apparently active only when linked with protein, whereas our yeast product is evidently equally active when protein-free, since it resists boiling and autoclaving and is active after precipitation of its protein content until it gives a negative biuret test.

Summary

1. Tests of the proliferation-promoting effects on yeast of intercellular wound hormones produced by the injury of newt, chick and rat tissues with lethal ultraviolet irradiation showed that the factors are active on yeast and can be assayed by quantitative yeast growth methods.

2. The data indicated that growth factors produced as a result of mechanical injury must be taken into account in considering results with animal tissue products. When the tissues were carefully washed after mincing, lethal ultraviolet irradiation led to fluids with potencies 2 to 9 times as great as those of control fluids from non-irradiated tissues. The active factor was stable to drying and autoclaving, and was not removed by filtration through Berkefeld filters.

3. Absorption spectra of the active preparations showed a maximum near 2600 Å. characteristic of purines and pyrimidines of the nucleic acid complex. The extinction at this wave-length closely paralleled the potency in various preparations. Tests of 15 amino-acids, yeast and thymus nucleic acids, inositol, aneurin, riboflavin, adenosine and insulin showed that all had either undetectable activity, or insufficient activity to account for that of the intercellular wound hormone preparations. It is concluded that the active factor in animal tissue preparations is either identical with or similar to that in yeast preparations (which contains adenine, guanine, pentose and phosphorus and is free from protein, pyridine or sulphur).

REFERENCES

Bonner & English (1937). *Science*, **86**, 352; and subsequent publications.
Caspersson (1936). *Skand. Arch. Physiol.* **73**, Supplement No. 8.
—— (1939). *Nature, Lond.*, **143**, 602.
Claude (1939). *Science*, **90**, 213.
Cook, Hart & Joly (1939). *Amer. J. Cancer*, **35**, 543.
—— Kreke & Nutini (1938). *Studies Inst. Divi Thomae*, **2**, 23.
—— Loofbourow & Stimson (1938). Xth Int. Congress of Chem., Rome, Italy, April 1938. To be published in the Acts of the Congress.
Davis (1937). *Can. med. Ass. J.* **36**, 237.
Fardon, Norris, Loofbourow & Ruddy (1937). *Nature, Lond.*, **139**, 589.
—— & Sullivan (1938). *Studies Inst. Divi Thomae*, **2**, 39.
—— —— & Andrus (1939). *Studies Inst. Divi Thomae*, **2**, 233.
Fischer (1930). *Virchows Arch.* **279**, 94.
—— (1939). *Nature, Lond.*, **133**, 113.
Haberlandt (1921). *Beitr. allg. Bot.* **2**, 1; and subsequent publications.
Harker (1938). *J. Cancer Res. Comm.* Univ. Sydney, **8**, 95.
Heyroth & Loofbourow (1931). *J. Amer. Chem. Soc.* **53**, 3441.
—— —— (1934). *J. Amer. Chem. Soc.* **56**, 1728.
Holiday (1936). *Biochem. J.* **30**, 1795.
Loofbourow, Cook, Dwyer & Hart (1939). *Nature, Lond.*, **144**, 553.
—— —— & Stimson (1938). *Nature, Lond.*, **142**, 573.
—— Cueto & Lane (1939). *Arch. exp. Zellforsch.* **22**, 607.
—— —— Whalen & Lane (1939). In the Press.
—— & Dwyer (1938, 1). *Science*, **88**, 191.
—— —— (1938, 2). *Studies Inst. Divi Thomae*, **2**, 129.
—— —— (1939). *Nature, Lond.*, **143**, 725.
—— —— & Morgan (1938). *Studies Inst. Divi Thomae*, **2**, 137.
—— & Morgan (1938). *Studies Inst. Divi Thomae*, **2**, 113.
—— —— (1939). *J. Bact.* **38** (in the Press).
Norris & Kreke (1937). *Studies Inst. Divi Thomae*, **1**, 137.
Orr (1935). *Brit. J. exp. Path.* **16**, 121.
Reader (1927). *Biochem. J.* **21**, 901.
Reiche (1924). *Z. Bot.* **16**, 241.
Schroeder & Cook (1939). *Studies Inst. Divi Thomae*, **2**, 247.
Sperti, Loofbourow & Dwyer (1937, 1). *Nature, Lond.*, **140**, 643.
—— —— —— (1937, 2). *Studies Inst. Divi Thomae*, **1**, 163.
—— —— & Lane (1937). *Science*, **86**, 611.
Törö (1937). *Z. mikr.-anat. Forschg.* p. 41; and subsequent publications.
Wiesner (1892). *Elementarstruktur*, Vienna, p. 102.
Wildiers (1901). *Cellule*, **18**, 313.
Williams, Lyman, Goodyear, Truesdail & Holaday (1933). *J. Amer. Chem. Soc.* **55**, 2912; and subsequent publications.

55. THE OXIDATION OF PYRUVATE IN PIGEON BREAST MUSCLE

BY HANS ADOLF KREBS AND LEONARD VICTOR EGGLESTON

From the Department of Biochemistry, University of Sheffield

(*Received 1 February 1940*)

PYRUVATE is very readily oxidized in animal tissues, yet little is known about the immediate products of its oxidation. Such oxidative reactions of pyruvate as are known to occur—dismutation, formation of succinate, acetate or ketone bodies—are side reactions whose significance varies from tissue to tissue: in no tissue can these reactions account for the total oxidation, and in some tissues, such as muscle or kidney, they account for even less than 20%.

We have tried to elucidate the oxidation of pyruvate in pigeon breast muscle by studying in detail the factors governing the oxidation. We find that fumarate acts as catalyst in the oxidation of pyruvate and that malonate breaks the catalysis when one molecule of pyruvate has reacted with one molecule of fumarate. The following reaction takes place in the presence of malonate:

(1) Fumarate + pyruvate + $2O_2$ = succinate + $3CO_2$ + H_2O.

In the absence of malonate this action is followed by the oxidation of succinate:

(2) Succinate + $\frac{1}{2}O_2$ = fumarate + H_2O.

The net effect of 1 and 2 is the complete oxidation of pyruvate:

Pyruvate + $2\frac{1}{2}O_2$ = $3CO_2$ + $2H_2O$.

In reaction 1 the succinate does not arise from fumarate by anaerobic reduction. It is formed from fumarate and pyruvate by a series of oxidative processes of the type formulated in the theory of the "citric acid cycle" [Krebs & Johnson, 1937, 1]. This theory is supported by the fact that a change of the experimental conditions directs reaction 1 in such a way as to yield citrate (about 15%) or α-ketoglutarate (about 50%) instead of succinate and CO_2. Up to the present the citric acid cycle is the only theory accounting for the experimental observations in pigeon breast muscle.

I. *Principle of the procedure*

Suspensions of minced muscle are shaken in Warburg manometer cups for periods varying between 20 min. and 4 hr., with and without pyruvate, fumarate, malonate or other substances. The disappearance of O_2 and pyruvate and the production of CO_2, succinate and other products are determined under varying conditions.

To make the quantities of the different metabolites directly comparable they are all expressed in terms of μl. gas, one μmol. being taken as equivalent to 22,400 μl.

Throughout the paper we write "fumarate" when in fact the system fumarate ⇌ *l*(+)malate is present, and "citrate" when in fact the system citrate ⇌ *cis*aconitate ⇌ *iso*citrate [see Johnson, 1939] is present.

Most of the results are presented in the form of tables, but of each series only a few experiments which we consider typical are included. The full experimental details are given in section XIX.

II. *Aerobic disappearance of pyruvate*

Minced pigeon breast muscle, suspended in phosphate saline and shaken in an atmosphere of O_2, removes added pyruvate with great rapidity. We find, for instance, that 4 ml. muscle suspension containing 267 mg. fresh muscle (or approximately 53 mg. dry tissue) remove 868 μl. or 3·4 mg. pyruvate in 1 hr. The rate of pyruvate utilization gradually decreases with time (Fig. 1 and Table 1). It ceases in the course of the third or fourth hour of the experiment, at the same time as does the respiration of the muscle. Under similar conditions the rate of the anaerobic removal of pyruvate, due to dismutation [Krebs & Johnson, 1937, 2] or to reactions with triosephosphate [see Meyerhof & Kiessling, 1935] amounts to no more than 5–10 % of the aerobic removal.

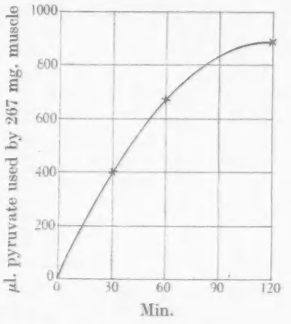


Fig. 1. Pyruvate utilization in minced pigeon breast muscle. (For details see Table 1.)

The best medium for experiments on the utilization of pyruvate is calcium-free phosphate saline. Plain phosphate buffer as used in previous experiments [Krebs & Eggleston, 1938] is definitely inferior, while phosphate buffer containing 0·01–0·06 M $MgCl_2$ is only slightly inferior to phosphate saline. Calcium when present in concentrations occurring in blood serum strongly inhibits the removal of pyruvate. These results are similar to those of Stare & Baumann [1936], Greville [1937] and Elsden [1939] who studied the effects of various media on the respiration of muscle. Examples and further experimental details are shown in Table 1.

Table 1. *Pyruvate utilization in pigeon breast muscle*

40°; O_2; 267 mg. fresh tissue in 4 ml. medium. The braces indicate sets of experiments carried out on the same muscle suspension and therefore directly comparable.

Medium	Period of incubation min.	Initial pyruvate in medium µl.	Final pyruvate in medium µl.	Pyruvate used µl.
Phosphate saline	120	1752	648	1104
" "	60	1716	974	742
{ " "	30	1708	1308	400
{ " "	60	1708	1030	678
{ " "	120	1708	820	888
{ Phosphate buffer (0·1 M; pH 7·4)	80	1734	1404	330
{ " " (0·1 M; pH 7·4) + 0·0025 M $MgCl_2$	80	1734	1184	550
{ " " (0·1 M; pH 7·4) + 0·005 M $MgCl_2$	80	1734	1120	614
{ Phosphate saline	80	1734	1005	729
{ Phosphate saline 0·0025 M $CaCl_2$	80	1734	1655	79

III. *O_2 consumption in the presence of pyruvate*

The O_2 consumption of muscle suspensions usually increases when pyruvate is added. The increase is small in the early periods of the experiment; it becomes more pronounced after 30 or 60 min. when the initial rate of respiration begins to fall in the control experiments. This fall is delayed by pyruvate as shown in Fig. 2 and Table 2.

Table 2. *O_2 consumption of minced pigeon breast muscle in the presence of pyruvate*

Suspension used	Conc. of pyruvate	μl. O_2 used after 30 min.	60 min.	120 min.
267 mg. muscle in 4 ml. phosphate saline	0·02 *M*	860	1665	2870
	0	815	1412	2165
267 mg. muscle in 4 ml. phosphate saline	0·02 *M*	1000	1885	—
	0	780	1270	—
267 mg. muscle in 4 ml. phosphate saline	0-02M	890	1552	1725
	0	740	965	1015

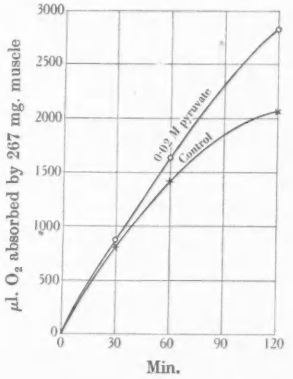


Fig. 2. O_2 consumption in the presence of pyruvate. (For details see Table 2.)

IV. *CO_2 production and respiratory quotient*

The CO_2 production increases even more than the O_2 uptake when pyruvate is added. Consequently the R.Q. rises, for example from 0·922 to 1·184 (Table 3).

Table 3. *Respiratory quotient in the presence of pyruvate*

4 ml. muscle suspension (267 mg. fresh muscle in phosphate saline; 40°); period: 60 min.; method of Warburg & Yabusoe [1924].

	No substrate added	0·02 *M* pyruvate added
μl. pyruvate used	—	742
μl. O_2 used	1270	1885
μl. CO_2 formed	1177	2237
R.Q.	0·922	1·184
Ratio $\frac{O_2 \text{ used}}{\text{Pyruvate used}}$	—	2·54
Ratio $\frac{CO_2 \text{ formed}}{\text{Pyruvate used}}$	—	3·01

The latter figure, being very close to 1·20 (i.e. the quotient calculated for the oxidation of pyruvate), suggests that pyruvate, if available, is the only substrate undergoing oxidation and this conclusion is borne out by the determination of pyruvate: for each mol. of pyruvate 2·54 mol. O_2 are absorbed (calculated 2·50) and 3·01 mol. CO_2 are liberated (calculated 3·0). These figures, it should be noted, represent the total gas exchange of the tissue. The extra gas exchange

caused by the addition of pyruvate is much too small to account for the removal of the pyruvate and we must therefore conclude that the added pyruvate competes for the O_2 with the substrates normally present in the tissue and suppresses their oxidation. In section XII we shall refer again to this inhibitory effect of pyruvate on the oxidation of other substrates when we discuss experiments in which pyruvate is used as an inhibitor of the oxidation of citrate and α-ketoglutarate.

V. *Effect of malonate*

Malonate inhibits the aerobic utilization of pyruvate (Table 4). The utilization is reduced to 8% by 0·025 *M* malonate and to about 23% by 0·001 *M* malonate. Most, if not all, of the removal of pyruvate remaining in the presence of 0·025 *M* malonate is due to anaerobic reactions which are not appreciably inhibited by malonate (see Table 4).

Table 4. *Effect of malonate on the removal of pyruvate*

4 ml. muscle suspension (267 mg. in phosphate saline); pyruvate added from side arm after 10 min., malonate added from the start; initial pyruvate concentration 0·02 *M*; 40°.

Malonate added final conc.	Gas	μl. pyruvate used after 1 hr.	μl. pyruvate used after 3 hr.
0	O_2	556	793
0·001 *M*	O_2	40	184
0·025 *M*	O_2	40	38
0	N_2	—	84
0·001 *M*	N_2	—	94
0·025 *M*	N_2	—	52

VI. *Effect of fumarate*

Fumarate abolishes the inhibitory effects of malonate. The extent of the fumarate effect depends on the relative concentrations of malonate and of fumarate: if the malonate concentration is low (approximately 0·001 *M*) its

Table 5. *Effect of fumarate on the removal of pyruvate at low and high concentrations of malonate*

4 ml. muscle suspension; 0·02 *M* pyruvate; pyruvate and fumarate added from side arm 10 min. after the addition of malonate.

Further substances added (final conc.)	Absolute quantity of fumarate in μl.	μl. pyruvate used after (min.) 30	60	120	180	μl. extra pyruvate used on addition of fumarate
Exp. I. Low malonate concentrations						
None	—	—	556	—	793	—
0·0025 *M* fumarate	56	—	672	—	928	135
0·001 *M* malonate	—	—	40	—	184	—
0·001 *M* malonate; 0·000625 *M* fumarate	56	—	436	—	492	308
0·001 *M* malonate; 0·00125 *M* fumarate	112	—	578	—	768	584
0·001 *M* malonate; 0·0025 *M* fumarate	224	—	666	—	928	744
Exp. II. High malonate concentration						
None	—	456	868	1028	1046	—
0·0025 *M* fumarate	224	452	740	1044	1005	—
0·025 *M* malonate	—	—	76	80	92	—
0·025 *M* malonate; 0·0025 *M* fumarate	224	168	244	292	313	221

inhibitory effect is completely removed by the addition of 0·0025 *M* fumarate. If the malonate concentration is high (approximately 0·025 *M*) addition of fumarate only restores the utilization of an equivalent quantity of pyruvate. These facts which are of decisive importance for the theory of pyruvate oxidation are illustrated by Table 5.

In the first experiment (Table 5) the inhibition caused by 0·001 *M* malonate is completely removed by 0·0025 *M* fumarate, but lower concentrations of fumarate have also great effects on the pyruvate utilization. This becomes clear when the quantity of fumarate present is compared with the effect produced: 1 mol. of fumarate causes an extra utilization of more than 5 mol. of pyruvate when the fumarate concentration is 0·000625 *M*.

In the second experiment (Table 5) where the malonate concentration is 0·025 *M* the addition of 224 μl. fumarate causes an additional pyruvate consumption of 221 μl. Further examples showing the equivalence of the amounts of fumarate added and pyruvate removed are given in Table 6. At high concentrations of fumarate the pyruvate removal is smaller than expected (because

Table 6. *Effect of fumarate on the disappearance of pyruvate in the presence of malonate*

O_2; 4 ml. muscle suspension; pyruvate added: 896 μl.

Concentration of malonate *M*	Time min.	μl. pyruvate used (in the absence of fumarate)	μl. fumarate added	μl. pyruvate used in the presence of fumarate	Extra pyruvate used / Fumarate added
0·0125	100	158	112	294	$\frac{136}{112}=1{\cdot}21$
0·0125	100	158	224	398	$\frac{240}{224}=1{\cdot}07$
0·0125	100	158	448	642	$\frac{484}{448}=1{\cdot}08$
0·025	80	27	56	78	$\frac{51}{56}=0{\cdot}91$
0·025	80	28	112	174	$\frac{146}{112}=1{\cdot}31$
0·05	120	130	224	299	$\frac{169}{224}=0{\cdot}75$
0·05	120	130	448	449	$\frac{319}{448}=0{\cdot}71$
0·05	120	130	896	499	$\frac{369}{896}=0{\cdot}41$
0·01	170	172	224	413	$\frac{241}{224}=1{\cdot}08$
0·01	170	172	448	592	$\frac{420}{448}=0{\cdot}94$
0·01	170	172	896	564	$\frac{392}{896}=0{\cdot}44$
0·01	115	99	56	165	$\frac{66}{56}=1{\cdot}18$
0·01	115	99	112	229	$\frac{130}{112}=1{\cdot}16$
0·01	115	99	224	380	$\frac{281}{224}=1{\cdot}25$
0·01	115	99	448	595	$\frac{496}{448}=1{\cdot}11$

of the gradual disappearance of the respiration in the experiments which makes it impossible to continue them over sufficiently long periods).

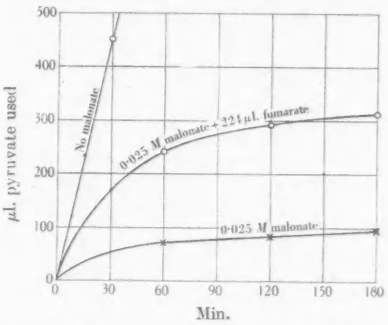


Fig. 3. Effect of malonate and fumarate on pyruvate utilization. Fumarate restores the utilization of an equivalent quantity of pyruvate. (For details see Table 6.)

The experiments recorded in Table 5 represent the two extreme, and therefore clear-cut types, of malonate inhibition. If intermediate concentrations of malonate (0·005 *M*) are used, a rapid oxidation of 1 mol. of pyruvate is followed by a slow disappearance of further molecules.

VII. *O_2 uptake in the presence of fumarate*

Szent-Györgyi has shown that fumarate increases the O_2 usage of muscle suspensions, especially in the presence of malonate. When the malonate concentration is low (0·001 *M*) the additional O_2 uptake caused by fumarate bears no quantitative relation to the amount of fumarate added. Fumarate acts like a catalyst and the extra O_2 uptake may therefore amount to a multiple of the quantity required for its complete oxidation. The position is however entirely different when the concentration of malonate is high (0·0025 *M*). In this case we find the additional O_2 uptake caused by fumarate to be proportional to the quantity of fumarate added. About 2–2½ mol. O_2 are taken up per mol. of fumarate (Table 7).

Table 7. *O_2 uptake in the presence of fumarate and malonate*

4 ml. muscle suspension.

Substrates added	μl. O_2 absorbed after					Extra O_2 absorbed / Fumarate added
Exp. I. (0·025 *M* malonate):	20 min.	40 min.	60 min.	80 min.		
None	69	107	129	156		—
896 μl. pyruvate	77	116	139	163		—
112 μl. fumarate	268	351	384	412		2·29
896 μl. pyruvate; 112 μl. fumarate	240	328	359	384		1·97
56 μl. fumarate	167	221	245	263		1·92
896 μl. pyruvate; 56 μl. fumarate	161	213	237	257		1·68
Exp. II. (0·01 *M* malonate):	20 min.	45 min.	65 min.	85 min.	115 min.	
869 μl. pyruvate	35	45	62	77	102	—
869 μl. pyruvate; 56 μl. fumarate	136	198	205	222	247	2·59
869 μl. pyruvate; 112 μl. fumarate	199	324	356	380	411	2·76
869 μl. pyruvate; 224 μl. fumarate	287	571	669	717	756	2·91
869 μl. pyruvate; 448 μl. fumarate	299	728	987	1180	1341	2·77

Since the complete oxidation of fumarate requires 3 mol. O_2 the oxidation of fumarate in the presence of 0·025 *M* malonate is incomplete. This incomplete oxidation, as will be shown later, can be formulated as follows:

(3) Fumarate + about $2O_2 + x \rightarrow$ succinate + about $2CO_2$,

where x is pyruvate, if available, otherwise an endogenous substrate such as lactate, or another triose derivative.

VIII. *Formation of succinate from fumarate*

As was first shown by Szent-Györgyi, succinate accumulates in respiring muscle poisoned by malonate. The amount of succinate formed is small when no substrate is added to the muscle; it increases slightly when pyruvate is added [Krebs & Johnson, 1937, 2; Weil-Malherbe, 1937] and rises very considerably when fumarate is added [Szent-Györgyi, 1935].

We found by varying the experimental conditions that the yield of succinate after addition of fumarate can be brought up to 80–100 % (in terms of the fumarate added). The highest yields of succinate were obtained if fumarate and pyruvate were added together and if the solution was analysed after the absorption of approximately 2–3 mol. O_2 per mol. of fumarate. An example and further details are given in Table 8.

Table 8. *Formation of succinate from fumarate and pyruvate in the presence of malonate*

0·0125 *M* malonate; 4 ml. muscle suspension; O_2 in Nos. 1–8; N_2 in No. 9.

	1	2	3	4	5	6	7	8	9
Substrates added		840 µl. pyruvate	112 µl. fumarate	840 µl. pyruvate 112 µl. fumarate	224 µl. fumarate	840 µl. pyruvate 224 µl. fumarate	448 µl. fumarate	840 µl. pyruvate 448 µl. fumarate	840 µl. pyruvate 448 µl. fumarate
µl. O_2 absorbed after									
20 min.	52	57	286	286	415	462	445	424	Anaerobic conditions
40 min.	83	88	377	370	635	603	820	805	—
60 min.	100	107	416	406	731	689	1020	995	—
80 min.	114	121	—	—	—	—	1070	1035	—
µl. pyruvate found	—	716	—	562	—	450	—	278	732
µl. pyruvate used	—	124	—	278	—	390	—	562	108
µl. pyruvate used due to added fumarate	—	—	—	154	—	266	—	438	—
µl. succinate found	18	29	103	140	186	230	286	392	94
µl. succinate due to added fumarate	—	—	85	111	164	201	264	363	—
Fumarate added/ extra pyruvate used/ extra succinate formed				1/1·35/0·99		1/1·19/0·90		1/0·98/0·81	

In interpreting these findings one must bear in mind that a direct reduction of fumarate to succinate is inhibited by malonate (see Table 8, anaerobic experiment). Hence the conversion of fumarate into succinate in the presence of malonate cannot be explained by an anaerobic reduction and it follows that there must be a second mechanism (which is oxidative, and unaffected by malonate) whereby fumarate is converted into succinate.

Before discussing the nature of this mechanism, we report an experiment in which another of the end-products of reaction 3, viz. CO_2, was quantitatively determined.

IX. *CO_2 production in the presence of fumarate and malonate*

In this experiment the R.Q. was measured in the presence of malonate by Warburg & Yabusoe's [1924] method. At the same time the decrease in pyruvate and the increase in succinate were determined. The results are shown in Table 9.

Table 9. *R.Q., pyruvate usage and succinate production in the presence of malonate (0·025 M)*

4 ml. muscle suspension; 100 min.

No.	Substrates added	μl. O_2 used	μl. CO_2 formed	μl. pyruvate used	μl. succinate formed
1	224 μl. fumarate	686	736	—	149
2	886 μl. pyruvate	129	110	25	20
3	224 μl. fumarate + 896 μl. pyruvate	606	778	165	207
	Metabolism due to addition of 10^{-2} m*M* fumarate in the presence of pyruvate (obtained by deduction of No. 2 from No. 3)	476 ($2{\cdot}13 \times 10^{-2}$ m*M*)	668 ($2{\cdot}98 \times 10^{-2}$ m*M*)	140 ($0{\cdot}63 \times 10^{-2}$ m*M*)	187 ($0{\cdot}84 \times 10^{-2}$ m*M*)

Per mol. of fumarate added 2·13 mol. of O_2 and 0·63 mol. of pyruvate are used, and 2·98 mol. of CO_2 and 0·84 mol. of succinate are formed. These results together with those reported in the preceding sections may be summed up with reasonable approximation by the equation

$$(1)\quad \text{Fumarate} + \text{pyruvate} + 2O_2 = \text{succinate} + 3CO_2 + H_2O.$$

The deviations from this equation which occur in some experiments may be explained either by side reactions, viz.

$$(4)\quad \text{Fumarate} + \tfrac{1}{2}O_2 \rightarrow \text{oxaloacetate} \rightarrow \text{pyruvate} + CO_2,$$
$$(5)\quad \text{Lactate (or triose)} + \tfrac{1}{2}O_2 \rightarrow \text{pyruvate},$$

or by the incomplete inhibition of succinic dehydrogenase. The latter would permit the oxidation of succinate and the repetition of reaction 1. This will occur when the concentration of malonate is comparatively low.

X. *Theory of the aerobic formation of succinate from fumarate*

At this stage it may be of use for the guidance of further experiments to discuss the theory of the reactions described in the preceding section. Szent-Györgyi [1935], who observed the aerobic formation of succinate from fumarate in the presence of malonate and realized that this could not be explained by the anaerobic reduction of fumarate, proposed the hypothesis of the "over-reduction" of oxaloacetate. He assumed that fumarate is first oxidized to oxaloacetate and that the latter is then "over-reduced" to succinate:

$$\text{Fumarate} \xrightarrow{+\frac{1}{2}O_2} \text{oxaloacetate.}$$
$$\text{Oxaloacetate} \xrightarrow{\text{"over-reduction"}} \text{succinate.}$$

"Over-reduction" is defined as a process by which oxaloacetate is directly reduced to succinate without the intermediation of fumarate or malate. Since this is an *ad hoc* hypothesis for which no evidence whatsoever has as yet been adduced, it cannot be regarded as a satisfactory explanation.

Another explanation is contained in the theory of the citric acid cycle [Krebs & Johnson, 1937, 1] which postulates the following reactions:

$$(6)\quad \text{Fumarate} \xrightarrow{+O} \text{oxaloacetate} \xrightarrow{+\text{pyruvate}+O} \text{citrate} + CO_2 \xrightarrow{+O} \alpha\text{-ketoglutarate} + CO_2 \xrightarrow{+O} \text{succinate} + CO_2.$$

The net effect of this series is equation 1.

When this theory was first proposed [Krebs & Johnson, 1937, 1] it was based on three different observations. The first showed that oxaloacetate yields succinate in the presence of O_2 and malonate, i.e. under conditions preventing the anaerobic reduction. The second demonstrated the formation of citrate from oxaloacetate under anaerobic conditions. The third showed that the oxidation of citrate to α-ketoglutarate and succinate is very rapid and that it is therefore permissible to ascribe to these substances the role of intermediate metabolites.

Of these three observations the first was the most important because it was incompatible with Szent-Györgyi's theory of the catalytic function of the system

$$\text{Fumarate} \rightleftharpoons \text{oxaloacetate}.$$

It postulated a new mechanism for the formation of succinate. The other two observations served to support the theory that the postulated reactions are those formulated in the citric acid cycle. In themselves these two observations did not constitute direct evidence in favour of the occurrence of the cycle in normal respiration, since they could also be interpreted as side reactions of minor importance. The first, however, proved conclusively that there is an oxidative reaction leading to the formation of succinate.

This is confirmed by the experiments reported in the preceding sections of this paper and summarized by reaction 1. The citric acid cycle is so far the only theory which explains reaction 1. At the same time there is no observation which contradicts the theory. Scheme 6 is further supported by the fact that the conclusions drawn from it are confirmed by experiment as will be shown in the next sections.

XI. *Succinate formation from further substances in the presence of malonate*

If scheme 6 is correct, the intermediate stages between fumarate and pyruvate on the one side, and succinate on the other, should all form succinate under the conditions in which reaction 1 is found to take place. That this is qualitatively the case is already known from earlier papers by Szent-Györgyi [1935], Martius & Knoop [1937] and Krebs & Johnson [1937, 1], but the previous experiments do not yet answer the quantitative aspect of this postulate, according to which the yields of succinate must be no less, and rates of its formation no lower than those found in the presence of fumarate and pyruvate. To fill in this gap we have carried out further experiments (Table 10) which show that the rates of the postulated reactions, as measured by the rates of O_2 absorption and the yields of succinate, are of the expected order. This of course does not prove that the reactions are actually intermediate stages. Theoretically it remains possible that there are alternative routes, but no other path is as yet supported by experimental evidence. We have tested those substances which might possibly be expected to be alternative intermediates in reaction 1, namely acetate, acetoacetate, aspartate, citramalate, citraconate, mesaconate, alanine, α-hydroxyglutarate, β-hydroxybutyrate, tiglate, crotonate, acrylate, and none of them

Table 10. *Succinate formation and O_2 uptake in the presence of malonate and various substrates*

267 mg. muscle in 4 ml. phosphate buffer (0·1 *M*; *p*H 7·4) containing 0·005 *M* $MgCl_2$ and 0·01 *M* malonate.

No.	Substrates added	µl. Succinate formed	µl. O_2 absorbed
1	None	37	118
2	224 µl. fumarate 896 µl. pyruvate	226	640
3	224 µl. oxaloacetate 896 µl. pyruvate	172*	439
4	224 µl. citrate	204	280
5	224 µl. α-ketoglutarate	—	214

* The lower yield of succinate in the presence of oxaloacetate can be explained by the side reaction 4.

was found to behave like an intermediate. Either they are not oxidized at all, or they fail to yield succinate in the presence of malonate. Little can therefore be said at present for alternative theories.

XII. *Formation of α-ketoglutarate and citrate*

To test the validity of scheme 6 in another and more direct way we tried to break this series of reactions at stages prior to the formation of succinate. This was achieved by making use of the competitive inhibition by pyruvate mentioned in section IV of this paper. Pyruvate, as was shown in section IV, is preferentially oxidized when several substrates are present; in other words it suppresses the oxidation of other substrates and an excess of pyruvate would therefore be expected to cause an accumulation of intermediates. The experiments show that this is the case if fumarate together with an excess of pyruvate is added. α-Ketoglutarate appeared in quantities reaching about 50 % of the maximum to be expected and citrate in quantities reaching about 15 %. The details describing the conditions under which these yields are obtained are given in Tables 11–14.

Table 11. *Effect of pyruvate on the accumulation of α-ketoglutarate in the presence of fumarate*

4 ml. muscle suspension; 60 min.; the yields are calculated in terms of fumarate added.

Substrates added	µl. O_2 used	µl. α-Ketoglutarate formed
None	993	0
0·01 *M* fumarate (=896 µl.)	1620	109 (12 % yield)
0·01 *M* fumarate; 0·01 *M* pyruvate	1460	204 (23 % yield)
0·01 *M* fumarate; 0·02 *M* pyruvate	1500	237 (26 % yield)

Table 12. *Time course of the accumulation of α-ketoglutarate in the presence of pyruvate and fumarate*

4 ml. muscle suspension; 0·0025 *M* fumarate (224 µl.); 0·02 *M* pyruvate; yields calculated as in Table 12.

Time min.	µl. O_2 used	µl. Succinate formed	µl. α-Ketoglutarate formed
30	740	0	114 (51 % yield)
60	1260	7	114 (51 % yield)
120	1670	17	118 (53 % yield)

LINDA HALL LIBRARY
KANSAS CITY, MO.

Table 13. *Effect of fumarate concentration on the formation of α-ketoglutarate*

4 ml. muscle suspension; 0·03 *M* pyruvate; 60 min.

Conc. of fumarate	μl. fumarate added to 4 ml.	μl. α-ketoglutarate formed
0·0025 *M*	224	132 (59% yield)
0·005 *M*	448	174 (39% yield)
0·01 *M*	896	272 (30% yield)

Table 14. *Formation of citrate in the presence of pyruvate and fumarate*

4 ml. muscle suspension; O_2.

Substrates added	Time of incubation min.	μl. citrate found	Yield of citrate as % of fumarate added
None	60	~0	—
0·02 *M* pyruvate	60	24	—
0·02 *M* pyruvate; 0·01 *M* fumarate	60	49	5·5
0·02 *M* pyruvate	40	4	—
0·02 *M* pyruvate	40	17	—
0·02 *M* pyruvate; 0·01 *M* fumarate	40	55	6·1
0·02 *M* pyruvate	90	7	—
0·02 *M* pyruvate; 0·0025 *M* fumarate	90	38	17·0
0·02 *M* pyruvate; 0·005 *M* fumarate	90	57	12·7
0·005 *M* fumarate	90	14	—

That fumarate must be added together with pyruvate becomes obvious when the nature of the "preferential oxidation" of pyruvate is considered in detail. The "preferential" oxidation applies in the first instance to the primary step of the oxidation, that is, according to the citric acid cycle, the reaction

$$\text{Oxaloacetate} + \text{pyruvate} \xrightarrow{+O} \text{citrate.}$$

This reaction will come to a standstill when all available oxaloacetate is used up. After this such other substrates as are available, for instance citrate, will undergo oxidation, and pyruvate will not be attacked again before the cycle has been completed. Since the concentration of oxaloacetate (or its precursors) is low in normal muscle, no appreciable accumulation of intermediates can be expected: pyruvate is oxidized to completion (Table 3). Only after addition of oxaloacetate (or its precursors) can accumulation of citrate and α-ketoglutarate take place. The experimental results are thus in perfect agreement with the theory.

XIII. *Aerobic formation of succinate from fumarate in the absence of added pyruvate*

Even when no pyruvate has been added to the tissue, fumarate yields, though in smaller quantities, succinate (Table 8), α-ketoglutarate (Table 11) and citrate (Table 14). We assume that these substances are formed in the same way, i.e. by reaction 1 in the presence and in the absence of added pyruvate and that the pyruvate required in reaction 1 is formed in the tissue from carbohydrate derivatives. Pyruvate is in fact formed with great rapidity when oxaloacetate is added anaerobically, as discovered by Szent-Györgyi [1936], according to the reaction:

(7) Oxaloacetate + "triosephosphate" → fumarate + pyruvate.

Recent work makes it probable that "triosephosphate" is 1:3-glyceraldehyde diphosphate [Negelein & Brömel, 1939].

The somewhat lower yield of succinate, referred to above, is probably due to the side reaction 4. This side reaction which causes a loss of the C_4-chain will be prevented when pyruvate is present in excess, as the pyruvate will react with the oxaloacetate at once when it is formed.

XIV. *The role of pyruvate oxidation in normal respiration*

The significance of reaction 1 depends on the part which it plays in the normal respiration of the tissue. It is therefore of interest to examine the relations between pyruvate oxidation and normal respiration; if the former is part of the latter, factors controlling the oxidation of pyruvate should also play a part in the normal respiration. Among these fumarate and malonate are the most conspicuous factors. It is already known from Szent-Györgyi's work that the normal respiration of pigeon breast muscle, like the oxidation of pyruvate, is inhibited by malonate and promoted by fumarate. At low concentrations of malonate (0·001 *M*) added fumarate acts as a catalyst and abolishes the malonate inhibition [see also Stare & Baumann, 1936]. Further experiments were however necessary to investigate the effect of fumarate quantitatively, especially at high concentrations of malonate. We find again a complete analogy between normal respiration and pyruvate oxidation: both reactions are *temporarily* restored by added fumarate. The period of the restoration is proportional to the quantity of fumarate added; it ends in the case of normal respiration when about 2·5 mol. per mol. of fumarate are absorbed. This is shown in Table 15 and in Fig. 4. It

Table 15. *Effect of fumarate on the O_2 uptake in the presence of malonate*

4 ml. muscle suspension; malonate concentration in Exp. I: 0·0125 *M*; in Exp. II: 0·025 *M*.

			Exp. I		
Quantity of fumarate added (μl.)	—	112	224	448	0 (No malonate)
μl. O_2 absorbed after					
10 min.	45	187	265	230	270
20 min.	77	304	516	530	540
40 min.	118	409	735	970	1065
70 min.	146	494	854	1255	1440
90 min.	164	526	915	1332	1650
100 min.	171	542	936	1368	1720
μl. succinate formed	32	132	161	262	0
			Exp. II		
Quantity of fumarate added (μl.)	—	56	112	224	—
μl. O_2 absorbed after					
20 min.	44	157	238	294	—
40 min.	72	206	320	501	—
60 min.	97	237	360	587	—
100 min.	138	296	440	665	—
μl. extra O_2 absorbed on addition of fumarate	—	148	302	527	—
$\frac{\text{Extra } O_2}{\text{Fumarate}}$	—	2·65	2·69	2·36	—

will be seen that the curve of the O_2 uptake bends sharply after the absorption of approximately 2·5 mol. of O_2 but continues to be a little steeper than the control without fumarate. The theory of malonate inhibition (see later) explains

this behaviour. The last column of Table 15 shows that the greater part of the added fumarate is converted into succinate in the course of the oxidation. It is further of importance that the rate of the restored oxidation is of the same order as that of normal respiration.

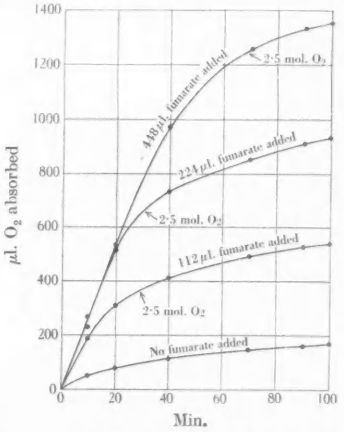


Fig. 4. Effect of fumarate on the O_2 uptake in the presence of malonate (0·0125 *M*). (For details see Table 15.)

The facts thus indicate that the oxidation of pyruvate, by way of reaction 1, is part of the normal respiration in pigeon muscle. It is of course the accepted view that carbohydrate is the chief substrate in muscle respiration and that pyruvate is an intermediate in the oxidation of carbohydrate, but it is a different matter whether the specific reaction 1 is part of the normal respiration. The comparison of this reaction and the total respiration permits the conclusion that reaction 1 represents in fact the bulk of the normal carbohydrate respiration. The latter only comprises two further reactions, namely:

$$C_3H_6O_3 \text{ (lactate, or triose or triosephosphate)} + O \rightarrow \text{pyruvate}$$

and

$$\text{Succinate} + \tfrac{1}{2}O_2 \rightarrow \text{fumarate}.$$

XV. *The evidence in favour of the citric acid cycle*

On the grounds of the facts discussed in section VIII we postulated that there is in muscle tissue an oxidative process whereby fumarate and oxaloacetate are converted into succinate. It has now been demonstrated that this process can be directed in such a way as to yield up to 50 % α-ketoglutarate or up to 15 % citrate. The high yield of the α-ketoglutarate must be considered as direct proof of its intermediate formation in reaction 1. As regards the intermediate formation of citrate the evidence is not so weighty, as the maximum yields could not be raised above 15 %. It may therefore be argued that only part of reaction 1 passes through the stage of citrate. This is however an unconvincing argument, since incomplete yields must be expected owing to the rapid oxidation of the intermediates under the experimental conditions. We therefore consider the evidence in favour of the citric acid cycle as complete as can be expected for this type of reaction scheme.

The theory is further supported by the fact that certain carbohydrate derivatives and the members of the citric acid cycle are the only substances (apart from aspartate and glutamate) which are known to be readily oxidized in muscle tissue, although more than 80 substances have been tested by various workers for their oxidation since Thunberg [1910] realized that this method may provide a suggestion as to whether a substance is an intermediate metabolite.

It is also of interest to recall that Batelli & Stern [1911] concluded from their experiments as early as 1911 that the oxidations of citrate, fumarate and malate form part of the chief respiratory process in muscle tissue. The metabolic relations between carbohydrate and these acids were necessarily obscure to Batelli & Stern, but the correctness of their conclusion is fully borne out by modern development.

When the theory was first formulated the compound reacting with oxaloacetate to form citrate was provisionally termed "triose" or "carbohydrate derivative" [Krebs & Johnson, 1937, 1]. In the second paper [Krebs, 1937, 1] "pyruvic acid (?)" was inserted. We can now omit the query since it has been shown that (*a*) pyruvate reacts in preference to other substances, (*b*) that such other substances as may react can be oxidized to pyruvate in the tissue.

At the present stage, it may be pointed out, the theory is no more than a skeleton scheme. Many details remain to be worked out, e.g. those concerning the synthesis of citrate or the role of phosphorylations. Some other details concerning further intermediates (*iso*citrate, *cis*aconitate, malate) and the role of the coenzymes have for brevity's sake been omitted from our considerations.

XVI. *Theory of the malonate inhibition*

Malonate is known to inhibit succinic dehydrogenase specifically [Thunberg, 1910; Quastel & Wooldridge, 1928; Gözsy & Szent-Györgyi, 1934]. The inhibition is competitive, i.e the degree of inhibition does not depend on the absolute concentration of malonate, but on the ratio succinate/malonate. Direct proof of the specificity of the inhibition in muscle tissue is the accumulation of succinate in the solutions. We find an inhibition of about 50% when the above ratio is 9·5. The concentration of succinate (including its precursors) in our muscle suspensions is below 0·0005 *M* when no substrate has been added (as measured by the amount of succinate accumulated in the presence of malonate). Hence 0·001 *M* malonate is expected to inhibit respiration more than 90%. When 0·0025 *M* fumarate is added (see Table 4) this will gradually be converted into succinate and the malonate inhibition of succinic dehydrogenase, though still about 50%, will be sufficiently overcome to provide the fumarate required for the continuation of the cycle at the full rate. If high concentrations of malonate (0·025 *M*) are employed, the succinate formed will not be able to overcome the malonate barrier sufficiently and when all the added fumarate is used up, the respiration will continue at a greatly reduced rate, depending on the amount of succinate oxidized. This explains the sharp bend in the curves of Fig. 4.

XVII. *Szent-Györgyi's cycle*

The theory of the citric acid cycle is not contradictory of but supplementary to Szent-Györgyi's theory, according to which the system:

$$\text{Fumarate} \rightleftharpoons \text{oxaloacetate},$$

acts as a hydrogen carrier in carbohydrate oxidation. The bases of this theory are the following observations of Szent-Györgyi and his collaborators:

1. The C_4-dicarboxylic acids promote respiration catalytically, i.e. without being used up.

LINDA HALL LIBRARY
KANSAS CITY, MO.

2. Respiration is inhibited by malonate.

3. Fumarate restores the respiration in the presence of malonate (as shown in this paper the restoration is incomplete when the malonate concentration is high).

4. Fumarate is rapidly oxidized to oxaloacetate, and oxaloacetate is rapidly reduced to fumarate.

5. "Triose" is the hydrogen donator for the reduction of oxaloacetate. "Triose" undergoes oxidation to pyruvate.

These facts (no. 3 with the important limitation stated above) have all been established beyond doubt. On the other hand it must be remembered that Szent-Györgyi never analysed in detail the mechanism of the oxidation of pyruvate in muscle tissue. In discussing the fate of the "hydrogen atoms of the substrates" he does not concern himself with the earlier stages of the hydrogen transport, i.e. with those reactions through which the hydrogen is released from the carbohydrate molecule (with the exception of reaction 7). The citric acid cycle thus fills a gap left open in Szent-Györgyi's work.

The oxidation of "triose" to pyruvate is certainly not the only reaction in which Szent-Györgyi's system acts as a hydrogen carrier. Several observations suggest that it plays a part in the reactions leading to the formation and oxidation of citrate. This remains to be investigated. The decisive argument against the view that Szent-Györgyi's theory fully explains the catalytic effects of fumarate is the fact that fumarate does not completely remove the malonate inhibition, but only restores a fraction of the respiration equivalent to the amount of fumarate added.

XVIII. *The citric acid cycle in other tissues*

Preliminary experiments on other tissues show that the citric acid cycle occurs in heart muscle and in kidney, and possibly in liver, but it seems that other mechanisms also play a significant role in the two latter tissues. It is doubtful however whether the citric acid cycle occurs in brain [cf. Banga *et al.* 1939] or in testis.

Experiments which show the occurrence of the citric acid cycle in cat kidney are also contained in a recent paper of Breusch [1939] who found a rapid synthesis of citrate from oxaloacetate and pyruvate under anaerobic conditions, and a rapid removal of added citrate under aerobic conditions.

XIX. *Experimental details*

1. *Muscle suspensions.* Pigeon breast muscle was cooled on ice immediately after the death of the animal and minced in the Latapie mill. Unless otherwise stated 4 g. (fresh weight) of muscle were suspended in 45 ml. ice-cooled phosphate saline, made up by mixing 1000 ml. 0·9% NaCl, 40 ml. 1·15% KCl, 10 ml. 3·84% $MgSO_4, 7H_2O$ and 300 ml. phosphate buffer, *p*H 7·4. The latter was prepared by dissolving 17·8 g. $Na_2HPO_4, 2H_2O$ and 20 ml. *N* HCl in 1 l. This medium, in accordance with the results of Stare & Baumann [1936] and of Greville [1937], was found to give higher metabolic rates than phosphate buffer + NaCl [Krebs & Eggleston, 1938] or phosphate buffer containing 0·006 *M* $MgCl_2$ [Greville, 1937].

3 ml. muscle suspension, measured with a wide-mouthed pipette, were placed in a conical manometric cup provided with a side arm and a centre chamber. Further additions (substrates, inhibitors, water) amounted to 1 ml., so that the final suspension contained 1 part of muscle in 15 parts of medium.

This degree of dilution was found to be the optimum for the work reported in this paper. Acidic substances were added in the form of neutral Na salts.

2. *Determination of pyruvate.* At the end of the experimental period 1 ml. 3 *M* acetate buffer, *p*H 5·0, was added to 4 ml. muscle suspension, to stop further reactions and to adjust the *p*H. Pyruvate was then determined manometrically by the carboxylase method in an aliquot of the filtrate [see Westerkamp, 1933; Krebs & Johnson, 1937, 2]. The results are expressed in μl. CO_2 liberated from pyruvate in the presence of carboxylase.

3. *Determination of the O_2 uptake.* The centre chamber of the manometric cup contained 0·2 ml. 2 *N* NaOH (unless otherwise stated), so that the O_2 uptake could be measured by the "direct" method. Since the O_2 uptake was generally very rapid, the rate of shaking was fast, 70–80 periods per min., amplitude 12 cm. Frequently the O_2 uptake exceeded the quantities which can be directly measured on the 300 mm. manometer scale. In these experiments a reading was taken when the level of the manometric fluid approached the bottom of the scale and sufficient air was then admitted to raise the pressure to the top of the scale. After a few minutes' equilibration a new reading was taken. The O_2 usage between these two readings was calculated by interpolation.

If the O_2 uptake was to be measured in the presence of added substrates, the latter were generally added from the side arm after the first reading.

4. *CO_2 production and respiratory quotient.* To determine the R.Q. the method of Warburg & Yabusoe [1924] requiring 3 cups (including 2 with two side arms) for each determination was used.

5. *Malonate inhibition.* The inhibitory effect of malonate has a certain induction period, especially when the concentration of malonate is low. To eliminate the lag, the tissue was incubated with malonate before the substrate was added, generally for 10 min. In filling the cups malonate was added directly to the muscle suspension whilst the substrate was placed in the side arm and mixed with the tissue after the first reading, i.e. after having been shaken in the water bath for 10 min.

6. *Succinate determination in the presence of malonate.* Succinic acid was determined manometrically with the aid of succinic dehydrogenase [Szent-Györgyi & Gözsy, 1936; Krebs, 1937, 2]. The muscle suspension was washed into a measuring cylinder with water, 1 ml. 10% Na_2WO_4 and 1 ml. 0·8 *N* H_2SO_4, made up to 15 ml. and filtered. An aliquot of the filtrate (about 12 ml.) was poured into a Kutscher-Steudel extractor together with 2 ml. 3% $KMnO_4$, 1 ml. 50% H_2SO_4, 5 ml. of a solution containing 50 g. $MnSO_4, 7H_2O$, 140 g. Na_2SO_4 and 15 ml. conc. H_2SO_4 per litre and placed for 5 min. in a boiling water bath. This serves to destroy the malonate which interferes with the determination of succinic acid. At the same time this treatment converts α-ketoglutarate into succinate and the results obtained are therefore the sum of succinate + α-ketoglutarate. Separate determinations of α-ketoglutarate and succinate [see Krebs, 1938] showed that at least 90% of the "succinate" found by the above method is actually succinate when malonate is used as an inhibitor. The conclusions drawn from these experiments are not affected by the fact that "succinate" included a certain fraction of α-ketoglutarate.

7. *Determination of succinate and α-ketoglutarate in the absence of malonate.* When both succinate and α-ketoglutarate were to be determined in the absence of malonate (section XII) duplicate experiments were set up, one for each analysis. In the first, succinate was determined after ethereal extraction from the deproteinized solution. The second cup was treated with $KMnO_4$ before the ethereal extraction, as described in the preceding paragraph. The results of the

succinate determination represent in this case the sum of succinate and α-ketoglutarate.

8. *Determination of citrate.* The method of Pucher *et al.* [1936] was used with the precautions described previously [Krebs & Johnson, 1937, 1]. Dioxan was used in place of pyridine as the colour stabilizer [Johnson, 1939].

Summary

1. Added pyruvate is readily oxidized by minced pigeon breast muscle. The oxidation of other substrates is inhibited when an excess of pyruvate is present. This inhibition is a "competitive inhibition".

2. The oxidation of pyruvate is inhibited by malonate.

3. Fumarate removes the malonate inhibition. The removal is complete when the malonate concentration is relatively low (0·001 *M*), but is incomplete when the malonate concentration is higher (0·025 *M*). In the latter case each molecule of added fumarate causes the removal of 1 mol. of pyruvate, whilst 2 mol. of O_2 are absorbed and 3 mol. of CO_2 produced, according to the equation:

(1) Pyruvate + fumarate + $2O_2$ = succinate + $3CO_2$ + H_2O.

4. The succinate formed in reaction 1 cannot arise by anaerobic reduction since this reaction is inhibited by malonate. Thus there must be a second route leading from fumarate to succinate which is oxidative and unaffected by malonate.

5. If an excess of pyruvate is added, together with fumarate, reaction 1 yields citrate, or α-ketoglutarate, instead of succinate:

(8) Pyruvate + fumarate + O_2 → α-ketoglutarate (yield up to 50 %).

(9) Pyruvate + fumarate + O_2 → citrate (yield up to 15 %).

6. When no pyruvate, but fumarate, is added to muscle in the presence of 0·025 *M* malonate, a reaction similar to 1 takes place:

(10) Fumarate + triose equivalent + $2\frac{1}{2}O_2$ = succinate + $3CO_2$ + $2H_2O$.

7. Reactions 1 and 10 represent the major part of the normal respiration in pigeon breast muscle.

8. Szent-Györgyi's theory of hydrogen transport by the system fumarate ⇌ oxaloacetate is accepted for the conversion of triose into pyruvate, the only reaction for which it has been proved. It is probable that this system also acts as a hydrogen carrier in the reactions which lead to the formation and to the breakdown of citrate. The theory fails however to explain the oxidation of pyruvate, because it does not account for the oxidative formation of succinate from fumarate and for the stoichiometric relations shown in reaction 1.

9. All observations are explained by the theory of the citric acid cycle which is not contradictory of but supplementary to Szent-Györgyi's theory. Reaction 1 shows that a series of reactions of the type formulated in the citric acid cycle occurs. The theory is directly supported by reactions 8 and 9. Whilst there is no doubt that the major part of muscle respiration goes through the citric acid cycle, the possibility of an alternative reaction is not excluded. This possibility is however purely theoretical and so far without any experimental support.

This work was aided by grants from the Rockefeller Foundation and the Medical Research Council.

REFERENCES

Banga, Ochoa & Peters (1939). *Biochem. J.* **33**, 1980.
Batelli & Stern (1911). *Biochem. Z.* **31**, 478.
Breusch (1939). *Biochem. J.* **33**, 1757.
Elsden (1939). *Biochem. J.* **33**, 1895.
Gözsy & Szent-Györgyi (1934). *Hoppe-Seyl. Z.* **224**, 1.
Greville (1937). *Biochem. J.* **31**, 2274.
Johnson (1939). *Biochem. J.* **33**, 1046.
Krebs (1937, 1). *Lancet*, **2**, 736.
—— (1937, 2). *Biochem. J.* **31**, 2095.
—— (1938). *Biochem. J.* **32**, 108.
—— & Eggleston (1938). *Biochem. J.* **32**, 913.
—— & Johnson (1937, 1). *Enzymologia*, **4**, 148.
—— —— (1937, 2). *Biochem. J.* **31**, 645.
Martius & Knoop (1937). *Hoppe-Seyl. Z.* **246**, 1; **247**, 104.
Meyerhof & Kiessling (1935). *Biochem. Z.* **283**, 83.
Negelein & Brömel (1939). *Biochem. Z.* **301**, 135.
Pucher, Sherman & Vickery (1936). *J. biol. Chem.* **113**, 235.
Quastel & Wooldridge (1928). *Biochem. J.* **22**, 689.
Stare & Baumann (1936). *Proc. Roy. Soc.* B, **121**, 338.
Szent-Györgyi (1935). *Hoppe-Seyl. Z.* **235**, 1.
—— (1936). *Hoppe-Seyl. Z.* **244**, 105.
—— & Gözsy (1935). *Hoppe-Seyl. Z.* **236**, 54.
Thunberg (1910). *Skand. Arch. Physiol.* **24**, 23.
Warburg & Yabusoe (1924). *Biochem. Z.* **146**, 380.
Weil-Malherbe (1937). *Biochem. J.* **31**, 299.
Westerkamp (1933). *Biochem. Z.* **263**, 239.

56. THE CITRIC ACID CYCLE

(A reply to the criticisms of F. L. BREUSCH and of J. THOMAS)

BY HANS ADOLF KREBS

From the Department of Biochemistry, The University of Sheffield

(*Received 1 February 1940*)

ALTHOUGH, in the view of the author, the preceding paper [Krebs & Eggleston, 1940] removes any doubt about the occurrence and significance of the citric acid cycle in pigeon breast muscle, it was thought desirable to clarify the situation, by discussing in full some of the criticisms of the theory which Breusch [1937; 1939] and Thomas [1939] have expressed in recent papers. Both authors admit the occurrence of the citric acid cycle, but believe it to be an unimportant side reaction. Breusch argued that citrate was formed only in experiments in which the concentration of oxaloacetate was unphysiologically high and that the citrate was not formed from oxaloacetate, but from some undefined impurity. The latter argument was based on the fact that the yield of citrate in his experiments never exceeded 2% of the oxaloacetate added. Thomas [1939] observed that under certain conditions 100 mg. of added oxaloacetate yielded 16–22 mg. malate and only 1·3–1·8 mg. citrate. Breusch [1939] published at the same time somewhat similar data. Both authors concluded that the formation of citrate was of no importance in the oxidation of carbohydrate, and that the catalytic function of the C_4-dicarboxylic acids was fully explained by the theory of Szent-Györgyi. This theory, it may be recalled, states, that the catalytic functions of the C_4-dicarboxylic acids is completely described by the reaction scheme

$$\text{fumarate} \underset{+2H}{\overset{-2H}{\rightleftarrows}} \text{oxaloacetate}.$$

In contrast, the theory of the citric acid cycle states that oxaloacetate further reacts in another way as follows:

oxaloacetate + pyruvate → citrate → α-ketoglutarate → succinate → fumarate.

The following comments are made in reply to the criticisms of Breusch and of Thomas.

I. *The crucial experiment*

Both authors disregard one of the most important experimental observations in the field, viz. the oxidative formation of succinate from oxaloacetate in the presence of malonate. Since this fact was first shown [Krebs & Johnson, 1937], we have found conditions in which the aerobic formation of succinate can be demonstrated in the most striking manner. When low concentrations of oxaloacetate are used, the inhibition of the anaerobic reduction of oxaloacetate to succinate is almost complete. As shown in Table 1 only 39 μl. succinate are formed from oxaloacetate by anaerobic reduction but 6·5 times this amount (254 μl.) is formed in the presence of O_2.

Table 1. *Formation of succinate from oxaloacetate and fumarate in the presence of malonate*

Each vessel had 4 ml. muscle suspension, containing 267 mg. fresh muscle in phosphate saline; malonate added 10 min. before the addition of the other substrates; malonate concentration in all vessels 0·025 *M*; 40°; 70 min.; for further details see the preceding paper; succinate was determined as described in § XIX, 6.

	(1)	(2)	(3)	(4)
Substrate added... (final conc.)	0·02 *M* pyruvate 0·005 *M* fumarate	0·02 *M* pyruvate 0·005 *M* oxaloacetate	0·02 *M* pyruvate 0·005 *M* fumarate	0·02 *M* pyruvate 0·005 *M* oxaloacetate
Gas	O_2	O_2	N_2	N_2
μl. O_2 absorbed	884	595	0	0
μl. succinate formed	*306*	*254*	*43*	*39*

Succinate thus arises in large amounts from oxaloacetate, although the direct reduction of oxaloacetate is virtually blocked. The same applies to succinate formation from fumarate (Table 1).

This is the crucial experiment because it remains unexplained by Szent-Györgyi's theory and calls unequivocally for a revision of this theory. It postulates an oxidative reaction leading from oxaloacetate to succinate.

II. *Synthesis of citrate*

The citric acid cycle (as yet the only theory which offers an explanation for the aerobic formation of succinate from fumarate and oxaloacetate) was supported by the following observations:

(1) Citrate can be formed in muscle.

(2) Citrate can be oxidized to succinate in muscle.

(3) The rates of these reactions are of the expected order of magnitude.

As was already mentioned, these results have been questioned by Breusch [1937; 1939] and by Thomas [1939] who advanced two arguments against the experiments on the synthesis of citrate. The first is that synthesis of citrate occurred only at "unphysiologically" high concentrations of oxaloacetate; the second, that the yields of citrate did not exceed 2% of the oxaloacetate added.

In reply it may be pointed out (1) that citrate is also formed when fumarate is added, i.e. when oxaloacetate gradually arises during the experiment in "physiological" concentrations, (2) that the yields of citrate under these conditions reach 15% (see the preceding paper). That the yields are not greater is due to the fact that the methods of separating the synthesis of citrate from its oxidation are still most imperfect. The rate and degree of *accumulation* of citrate are therefore only minimum values for the actual *formation* of citrate.

One of the reasons for the incomplete yields of citrate is an anaerobic reaction between oxaloacetate and citrate. Citrate disappears when oxaloacetate is added, as shown in Table 2. The conditions favouring the synthesis of citrate also accelerate its removal.

Table 2. *Anaerobic removal of citrate by oxaloacetate*

4 ml. muscle suspension containing 0·4 g. pigeon breast muscle in 4 ml. phosphate saline in each cup; N_2; 40°; procedure as in the preceding paper.

	(1)	(2)	(3)
μl. citrate added	448	448	448
μl. oxaloacetate added	448	896	—
μl. citrate recovered after 60 min. incubation	216	189	415
μl. change in citrate	*−234*	*−261*	*−33*

The reaction between oxaloacetate and citrate suggests that the Szent-Györgyi system acts as a hydrogen carrier in the oxidation of citrate (or *iso*-citrate), a point at present under investigation.

Oxidation of citrate

In his recent paper Breusch [1939, p. 1769] makes the statement that pigeon breast muscle "has only slight ability to break down citric acid" and he concludes that it cannot act as an intermediate metabolite. This statement is contradictory to the findings of numerous previous investigators [Thunberg, 1910; Batelli & Stern, 1911] including Breusch himself [1937] and Thomas [1939]. To refute Breusch's recent view, it will suffice to quote Breusch's own previous observation. He found [1937, p. 277, Table 17] that 20 ml. of muscle suspension (containing 2 g. of wet muscle) removed 20 mg. citrate in 60 min. This quantity requires 10,600 μl. O_2 for complete oxidation. Breusch found the actual uptake per hr. of 20 ml. suspension to be only about 5000 μl. O_2 (p. 270, Table 8, column 3). Thus the rate of citrate disappearance can easily account for the total respiration of the muscle.

It is obvious, because of the cyclic nature of the metabolic processes, that added citrate will not necessarily disappear from the muscle under all circumstances. In the normally respiring muscle the rates of the formation and of the oxidation of citrate, and of all the other members of the cycle, are approximately equal. Only under special conditions, which upset the physiological balance, can the ability of the tissue to oxidize citrate be demonstrated, e.g. when poisons (As_2O_3, malonate) or a large excess of citrate (0·05 M) have been added.

Miscellaneous

Several of the criticisms arise solely from a misinterpretation of the theory. Thomas as well as Breusch draw incorrect conclusions from the theory and argue against the theory when these conclusions are not confirmed by their experiments. This applies, for instance, to Thomas's statement that the formation of malate from oxaloacetate must be inhibited by malonate if the citric acid cycle is correct. In actual fact it was expressly stated that the reactions succinate $\rightleftharpoons$ fumarate $\rightleftharpoons$ malate $\rightleftharpoons$ oxaloacetate are reversible, and that oxaloacetate can be converted into the other C_4 dicarboxylic acids in two ways, either anaerobically, or aerobically, the first being unaffected by malonate. Thomas omitted the reversibility symbol ($\rightleftharpoons$) in his version of the theory (p. 232) and hence reaches incorrect conclusions. Breusch argues that fumarate must yield citrate, because fumarate yields oxaloacetate. But the theory only postulates the intermediate formation, and not the accumulation of citrate. The accumulation can only be expected under special conditions, such as those defined in the preceding paper.

Finally it may be pointed out that the O_2 uptake in many of Breusch's and of Thomas's experiments is only 20–50 % of the average figures in our experiments. This applies to the data given in Table 4 of Thomas's paper and Table 2 in Breusch's paper [1937]. The low values indicate that the experiments were carried out with tissue which had already lost the greater part of its metabolic activity; thus it is not surprising that the results were unsatisfactory. Not only did Thomas find no catalytic effect of citrate, but he was also unable to reproduce the catalytic effects of fumarate and succinate [Thomas, 1939, Table 4] demonstrated by Stare & Baumann [1936].

SUMMARY

The criticisms put forward by Breusch and by Thomas against the theory of the citric acid cycle are discussed.

REFERENCES

Batelli & Stern (1911). *Biochem. Z.* **31**, 478.
Breusch (1937). *Hoppe-Seyl. Z.* **250**, 262.
—— (1939). *Biochem. J.* **33**, 1757.
Krebs & Johnson (1937). *Enzymologia*, **4**, 148.
—— & Eggleston (1940). *Biochem. J.* **34**, 442.
Stare & Baumann (1936). *Proc. roy. Soc.* B, **121**, 338.
Thomas (1939). *Enzymologia*, **7**, 231.
Thunberg (1910). *Skand. Arch. Physiol.* **24**, 23.

LINDA HALL LIBRARY
KANSAS CITY, MO.

57. GLYCOLYSIS IN CELL-FREE EXTRACTS OF BRAIN

By A. GEIGER

From the Physiological Laboratory, The Hebrew University, Jerusalem

(*Received 30 December 1939*)

The elucidation of the intermediary processes in muscle glycolysis accomplished in recent years has been largely due to the fact that active cell-free extracts of muscle could be prepared. Most of the known intermediary products of muscle glycolysis have been isolated from such extracts. On the other hand our methods are hardly adequate for dealing with intermediary processes in living cells. Thus no substantial progress has been made in elucidating the intermediary processes of brain glycolysis, for in brain the destruction of cell structure causes complete loss of the glycolytic power. Extracts retain only a small fraction of the glucolytic power of the brain tissue and have therefore proved unsuitable for the examination of the intermediary products involved in brain glycolysis. It was shown recently [Geiger, 1939] that on cytolysis a powerful inhibitor of glycolysis and of yeast fermentation is liberated from brain tissue and it was assumed that the presence of this substance is responsible for the lack of glycolysis in brain extracts. It was also assumed that after eliminating the antiglycolytic substance contained in cytolysed brain, the extracts could be reactivated by addition of coenzymes.

By the procedure described below highly active brain extracts have been prepared. The glucolytic rate of these extracts surpasses by 4–7 times that of brain slices or pulp, and is about 50 times that of the former extracts.[1] After adding the coenzymes the extracts are active even in dilutions as high as 1 : 250.

The high activity of these brain extracts made possible the examination of a few intermediary processes of brain glucolysis. The results obtained on these extracts differ in many respects from those obtained on brain tissue. Part of the divergence is possibly due to the selective permeability of brain cells. Naturally, the reservations made in the interpretation of results obtained on muscle extracts also apply to the present case.

General methods

The analytical and preparative methods employed in these experiments were those described in a previous paper [Geiger, 1939]. Albino rats were used in most experiments. Pyruvic acid was estimated according to Case & Cook [1931]. Embden ester was prepared according to Ostern *et al.* [1936], Cori ester according to Cori *et al.* [1937]. The phosphoric esters were estimated by acid hydrolysis

[1] About 670 mg. lactic acid are formed by 100 g. of chopped fresh brain tissue in 1 hr. at 37°, and about 1100 mg. by the same weight of sliced brain. The maximum weight of lactic acid obtained by a corresponding amount of extract was 80 mg. As the work of Euler *et al.* [1936] was done on acetone-precipitated extracts it is difficult to compare their figures with those obtained on fresh extracts. A rough calculation shows, however, that in their experiments about 40 mg. of lactic acid were formed by an amount of extract corresponding to 100 g. of fresh brain.

according to Lohmann [1928]. In several cases the Ba salts of the phosphoric esters were precipitated. Lactic acid was determined chemically if not stated otherwise.

Preparation of active extracts

Cell-free extracts prepared with isotonic solutions had a very slight glucolytic activity. It was found, however, that from brain cytolysed with distilled water the inhibitory substance previously described did not pass into the extracts if the extraction was carried out at a slightly acid reaction. Upon testing these extracts no glucolytic activity was found even after adding all the known coenzymes of muscle glycolysis. The addition of boiled extracts of beer yeast, however, caused these extracts to glycolyse at a rate surpassing even that of brain slices. The following experiment illustrates this: Exp. 1. Four brain preparations were made: (*a*) Cerebrum of young albino rats macerated in a smooth mortar with 4 times its weight of Ringer solution. (*b*) Cerebrum macerated with 3 times its weight of distilled water and 1 part of 0·1 *M* phosphate of *p*H 5·5. (*c*) Cerebrum macerated with 4 parts of water and (*d*) cerebrum macerated with 4 parts of 0·5 % $NaHCO_3$. After 10 min. standing on ice (*b*), (*c*) and (*d*) were centrifuged. 2 ml. each of pulp (*a*) and of the extracts (*b*), (*c*) and (*d*) were made up in separate Erlenmeyer flasks with the necessary additions to 4 ml. After filling up with N_2 containing 5 % CO_2 they were incubated at 37° for 1 hr. while rocking in the water thermostat. The additions consisted of 0·2 ml. *M*/5 phosphate buffer of *p*H 7·2, 0·8 ml. of 1·3 % $NaHCO_3$, 50 mg. glucose or glycogen, 1 mg. Ap,[1] 2 mg. CrP, 2 mg. GSH, 2 mg. CoZ and 0·5 ml. of boiled yeast extract. No Mg was added. The yeast extract was made by macerating 1 part of air-dried beer yeast with 4 parts of water and by heating the centrifuged extract for 10 min. in a boiling water bath. The contents of each flask correspond to 0·4 g. of fresh brain. Table 1 shows the amount of lactic acid formed in each flask calculated for 5 ml. of extract (corresponding to 1 g. of fresh brain).

Table 1. *The effect of boiled yeast extract on glycolysis of different brain preparations*

	mg. lactic acid formed in 1 hr. at 37° by 5 ml. extract	
	From glucose g.	From glycogen g.
A. Brain pulp in isotonic NaCl	6·32	1·09
The same + boiled yeast extract	6·85	1·92
B. Brain extract in phosphate solution of *p*H 5·5	1·16	0·80
The same + boiled yeast extract	15·5	5·2
C. Water extract of brain	0·80	0·85
The same + boiled yeast extract	27·0	8·60
D. Brain extract in 0·5 % $NaHCO_3$	0·36	0·51
The same + boiled yeast extract	2·10	1·30
E. Boiled yeast extract + all coenzymes	0	0

From this experiment it became evident that some substances contained in boiled yeast extract activate brain glycolysis. These substances were also found to be present in smaller amounts in muscle and in still smaller amounts in brain.

[1] In the following pages Ap stands for adenosinetriphosphate, CrP for creatinephosphate, Hdp for hexosediphosphate, HMP for Robison ester, EE for Embden ester, CE for Cori ester, CoZ for cozymase, GSH for reduced glutathione.

It should be pointed out that the water extracts, although more active than the phosphate extracts, under the present conditions lose the greatest part of their glucolytic power after 30–40 min. incubation at 37°. It is shown below that by diluting the water extracts, a constant high rate of glucolysis can be obtained for about 1 hr. The activity of the phosphate extract remains fairly constant for a long period also if used in high concentration.

Acetone-dried preparations of brain extracts were made by pouring 1 part of extract into 10 parts of ice-cooled acetone. The precipitate was quickly filtered, washed with cold acetone and dried. It was invariably found that the extracts lost about 40–60 % of the activity after precipitation in acetone.

In all the following experiments the extracts were prepared by thoroughly grinding rat cerebrum in an ice-cooled mortar with 4 parts of cold water. The brain was finely ground before the addition of the water which was added in small portions with continuous grinding. One part of 0·1 *M* phosphate buffer of *p*H 5·5 was then added, mixed, let stand in ice for 10 min. and centrifuged. If phosphate-free extract was desired, the extraction was carried out with 4 parts of water. The extracts kept their activity on ice for 2–3 days. The glucolytic rate in the water extract is usually more than twice as high as in the phosphate extracts. A second extract of the brain pulp has a slight glucolytic activity, the third extract is inactive.

The effect of dilution of the extracts on the glucolytic rate. It was seen in the above experiment that the water extracts lose their high initial rate of glucolysis after a short incubation. On the other hand the acid-phosphate extracts had a slower but more constant glucolytic rate. Further addition of coenzymes and of bicarbonate to the water extracts could not restore their activity. It was found later that the water extracts still contain some of the glycolysis inhibitor and that the presence of this inhibitor is responsible for the quick decline of the glucolytic rates. On diluting the extracts, however, the inhibitor loses its activity and the extracts maintain a very high and constant rate of glucolysis for 1–1½ hr. at 37°. The optimum dilution under our experimental conditions was found to be 0·5 ml. of extract to 10 ml., corresponding to a dilution of 1 part of fresh brain to 100 ml. Under these conditions a maximum amount of 64 mg. of lactic acid was formed by 5 ml. of extract—corresponding to 1 g. of fresh brain—in 1 hr. at 37°. The average figure from 84 experiments was 46 mg., i.e. about 4 times the amount which can be formed by slices under the same conditions.

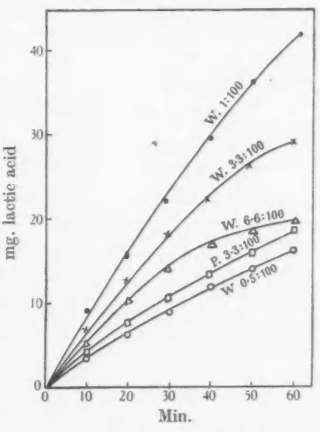


Fig. 1. The effect of dilution on the glucolytic rate in brain extracts. W, water extract; P, phosphate extract. Lactic acid is calculated in mg. for 1 g. of fresh brain. Dilutions are given in g. of fresh brain/ml. of fluid.

Further dilution of the extracts lowers the rate of glucolysis owing to the lack of a yet unknown coenzyme. The high dilution of the water extract has the further advantage that the concentration of coenzymes and of other active substances is very low. Experiments with different dilutions of brain extract are shown in Fig. 1.

Effect of dialysis on the activity of the extracts. For the experiments described in the following section it was desirable to free the extracts as far as possible from coenzymes and from preformed metabolites. On dialysis in collodion sacs and in cellophane tubes the extracts gradually lost their glucolytic power. After 8–10 hr. of dialysis a precipitate formed and the extract could not be reactivated by coenzymes and boiled yeast extract. The possibility has been considered that by dialysis a further coenzyme was eliminated. To test this possibility boiled extracts of muscle and of brain were concentrated in a vacuum and added to the dialysed extracts. In addition, fresh brain extract was dialysed and the dialysate concentrated in a vacuum. The addition of the concentrated boiled extracts and of the dialysate to dialysed brain extract did not reactivate the latter. It has to be assumed therefore that dialysis against distilled water inactivates the brain extracts irreversibly.

In later experiments it was possible to prevent the precipitation of the extracts and maintain a part of their activity by dialysis against 0·6% NaCl solution. After 24 hr. of dialysis only a slight precipitate formed and after the addition of coenzymes the extract still retained about a third of its original activity.

Coenzymes of brain glycolysis

(*a*) *Magnesium and hexosediphosphate.* From the experiment in Table 1, it is evident that the coenzymes contained in boiled yeast extract activate brain glycolysis. In attempting to separate the active principle from other components of the yeast extract the following procedure was adopted: air-dried beer yeast[1] was ground with 4 parts of water and centrifuged after standing 15 min. The extract was heated for 8 min. in a boiling water bath. To the cool extract Pb acetate was added until no more precipitate formed; it was then filtered. An excess of mercuric acetate was added to the filtrate and the precipitate was filtered off after a few hours. The filtrate was treated with H_2S and was concentrated under reduced pressure to a fifth of its original volume. After neutralization with $NaHCO_3$ a large excess of $NaHCO_3$ and enough $Ba(OH)_2$ were added to precipitate all the carbonate. The precipitate was filtered and washed with water. The active substance remained with the $BaCO_3$ precipitate. The $BaCO_3$ was then decomposed with just enough $5N$ H_2SO_4 and the $BaSO_4$ washed once with a small amount of water. It was thus possible to concentrate the active substance contained in 1 kg. of yeast into 50 ml. The filtrate was free from CoZ, Ap and GSH. For the sake of brevity further particulars are omitted as it was found later that the boiled yeast extract can be replaced by Mg and minute amounts of Hdp. The principal active component of the purified yeast extract was found to be Mg.

One of a large number of experiments is shown in Table 2. In these experiments 2 ml. of fresh phosphate extract of brain (corresponding to 0·4 g. of fresh brain) were diluted with the necessary additions to 10 ml. and incubated for 1 hr. at 37°. The lactic acid production is calculated as usual for 5 ml. of brain extract corresponding to 1 g. of fresh brain.

The inactivity of the extracts to which no Mg was added is surprising if the rather low dilution of the extracts is considered. In muscle extracts a relatively thorough dialysis is necessary before the lack of Mg becomes apparent. In order to test the optimum amount of Mg in brain extract, the following experiments were made with more diluted water-extract. 0·5 ml. of brain extract was made

[1] We are greatly indebted to the Rishon Brewery for kindly supplying us with fresh beer yeast.

Table 2. *The effects of Mg and of hexosediphosphate on glucolysis*

	mg. lactic acid formed by 5 ml. extract	
	From glucose	From glycogen
Brain extract + all coenzymes	0·80	0·55
The same + 0·5 ml. yeast extract	18·1	6·7
The same + 4 mg. $MgCl_2$	17·9	6·8
The same + 4 mg. $MgCl_2$ + yeast extract	18·1	6·7
The same + $MgCl_2$ + Hdp	18·3	6·8
The same + $CaCl_2$ + Hdp	0·9	0·45

up to 10 ml. The resulting dilution was 1 part of fresh brain to 100 parts of fluid. Other additions were: 2 mg. Ap; 1 mg. of CoZ; 3 mg. of GSH; 3 mg. of CrP; 0·5 mg. of Hdp; 1·8 ml. of 1·3% $NaHCO_3$; 0·8 ml. of a $M/5$ phosphate solution of *p*H 7·2 and 1 ml. of a 5% solution of glucose or glycogen. The results, calculated for 5 ml. of brain extract, are contained in Fig. 2.

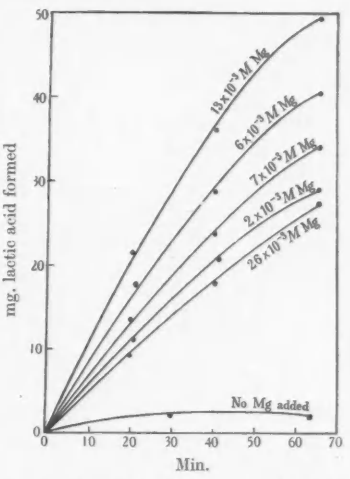


Fig. 2. The effect of Mg on the glucolytic rates in brain extract. Lactic acid is calculated for 1 g. of fresh brain. Dilution of the extract 1 : 100.

It appears that the concentration of Mg which is necessary for full activation of the brain extracts is about 3 times as high as that necessary for muscle extract. In our experiments the optimum concentration of Mg was about 13×10^{-3} M as compared with Lohmann's [1928] figures of $4{\cdot}8 \times 10^{-3}$ M for muscle extracts with glycogen and $1{\cdot}4 \times 10^{-4}$ M for Hdp. In these experiments lactic acid was estimated by chemical and by manometric methods. It was found, however, that higher Mg content made the manometric experiments unreliable. In these experiments some Mg phosphate was precipitated, and a large amount of CO_2 liberated, making measurements of glycolysis impossible. This CO_2 formation lasted for $\frac{1}{2}$–$\frac{3}{4}$ hr. It is interesting to note that addition of large amounts of coenzymes inhibited to a considerable extent the CO_2 formation and the precipitation of Mg phosphate.

Mg cannot be replaced by Ca. The addition of large amounts of Ca causes inhibition of glycolysis. Inhibition is caused also by larger amounts of Mg.

The addition of small amounts of Hdp further activated glucolysis in some experiments with highly diluted brain extract. The coenzymic function of Hdp in muscle glycolysis has been pointed out by Kendall & Stickland [1937]. Experiments with Hdp are also contained in Table 2. In all the following experiments the boiled extract of beer yeast was replaced by $MgCl_2$ and minute amounts of Hdp.

(*b*) *Creatinephosphate.* The role of CrP in muscle glycolysis has been extensively studied during recent years. It was found that its role as a coenzyme in muscle glycolysis is facultative [Parnas, 1937]. On the other hand, according

LINDA HALL LIBRARY
KANSAS CITY, MO.

to Meyerhof *et al.* [1937], for the main route of muscle glycolysis the presence of creatine is essential.

It seems, however, that in brain CrP is a coenzyme of glucose breakdown, although it is possibly not indispensable for lactic acid formation from glycogen. In the experiments on fresh extracts the effect of CrP on glucolysis was variable. In dialysed extracts, however, its effect was conclusive. We have not yet been able to find the reason for the variable effect of CrP in fresh extracts. In the majority of experiments with fresh extracts the activating effect of CrP was unmistakable while in some other experiments it was very small or not appreciable. For this reason a large number of experiments are reported in Table 3.

Attention should be drawn to the fact that in concentrated extracts the necessity of CrP is more evident than in the diluted extracts. In the majority of the experiments, if the extracts were diluted to 1 : 100, the maximal glycolytic rate was also obtained without the addition of CrP. In fresh water-extracts of brain the activating effect of CrP was very seldom visible.

In a number of experiments the extracts were autolysed without substrates at different reactions in order to split the preformed CrP. The behaviour of these extracts with regard to CrP was not different from that of the unautolysed extracts.

It should be pointed out that in no case had CrP any activating effect on lactic acid formation from glycogen. Dialysed extracts did not form any significant amount of lactic acid from glycogen.

CrP could not be substituted by creatine or by any of the known coenzymes.

Table 3. *The effect of phosphocreatine on glycolysis in brain extracts*

Dilution of the extract: 1 g. brain to total vol. (ml.)	mg. lactic acid formed by extracts corresponding to 1 g. of fresh brain, in 1 hr. at 37°			
	From glucose		From glycogen	
	Without CrP	With CrP	Without CrP	With CrP
A. Phosphate-extracts				
15	6·7	18·5	6·1	5·8
20	5·2	19·1	6·7	6·4
20	11·5	18·1	5·5	5·9
20	16·8	17·2	5·6	5·4
100	6·5	17·8	7·6	7·9
B. Water-extracts				
15	33·0	45·0	9·4	9·1
20	32·0	31·7	9·8	9·5
100	38·2	49·3	12·5	12·3
C. Dialysed water-extracts (for 24 hr. against 0·6 % NaCl)				
15	0·8	10·6	—	—
25	0·5	16·1	—	—
50	0·8	18·6	—	—

On the other hand the necessity of CrP in brain glucolysis could be conclusively demonstrated with dialysed extracts. For this purpose water extracts of brain were dialysed for 20–25 hr. against 0·6 % NaCl solution. The experiments made with such extracts have shown that in the absence of CrP no lactic acid is formed from glucose, mannose or fructose. All these experiments gave uniform results. The lactic acid formation from glycogen in dialysed extracts was always very low and these were unsuitable for the examination of the role of CrP in lactic acid formation from glycogen.

(*c*) *Adenosinetriphosphate.* It was shown [Geiger, 1935] that Ap activates glucolysis in brain pulp. This was confirmed by Euler *et al.* [1936]. In the present experiments it was seen that Ap is a coenzyme of brain glucolysis. In diluted extracts the glycolysis comes to a standstill after a very short incubation and is restored by the addition of Ap. In Fig. 3 one experiment taken from a larger number of identical ones is reported. In these experiments 0·5 ml. of water extract of brain was made up to 10 ml. with the usual additions.

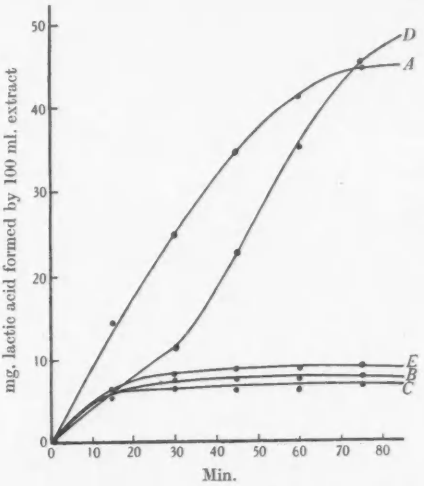


Fig. 3. The effect of cozymase, of Ap and of muscle adenylic acid. *A*, all coenzymes; *B*, without Ap; *C*, without cozymase; *D*, with adenylic acid and CrP, without Ap; *E*, with adenylic acid and phosphopyruvic acid without Ap.

The effect of Ap on glucolysis is evident even in concentrated brain extracts. Thus, in one experiment with 2 ml. of brain extract diluted to 3·5 ml., 24·6 mg. lactic acid were formed in 1 hr. with added Ap and 3·3 mg. without Ap. Under our experimental conditions about 1·5 mg. of Ap had to be added to 10 ml. in order to obtain a maximum activation.

Ap could not be replaced by yeast adenylic acid, but muscle adenylic acid together with CrP or with Hdp could replace it. In the latter case a long latent period was observed. Muscle adenylic acid with phosphopyruvic acid was less effective.

(*d*) *Cozymase.* Diphosphopyridinenucleotide (Euler) is indispensable in brain glycolysis. In all our experiments relatively large amounts of CoZ had to be added in order to obtain full activation.[1] In this respect the behaviour of brain extract was similar to that described by Boyland *et al.* [1937] for tumour extract. It is probable that the high CoZ requirement of brain extract is due to the rapid destruction of CoZ by an enzyme which is not necessarily involved in brain glycolysis. The experiment given in Fig. 3 was made similarly to those with

[1] The grade of purity of our cozymase preparation has not been tested. It was prepared according to Ohlmeyer [1936] and should be 90% pure.

Ap. For this experiment highly diluted extracts were used, but the effect of CoZ is very marked also in concentrated extracts.

(*e*) *Glutathione.* The activating effect of glutathione on brain extract has been described previously [Geiger, 1935]. As these earlier extracts had very slight activity we repeated the experiments on extracts made by our present method.

The main difficulty was encountered in obtaining extracts free from glutathione. Even after 25 hr. of dialysis the extracts still contain small amounts of glutathione. Activation of glucolysis by glutathione was seen in every experiment, but its degree was variable. The effect of glutathione on the extracts did not differ much from that on brain pulp. On the other hand dialysed extracts responded more regularly to glutathione. Euler *et al.* [1936] confirmed the activating effect of glutathione on brain glucolysis, but they attributed this action to the capacity of glutathione to combine with heavy metals. It was shown by Geiger & Magnes [1939] that this latter contention is untenable.

The results obtained with glutathione on brain extracts and on brain pulp are very similar to those of Needham & Lehmann [1937] obtained on embryo brei. Although it seems to be fairly well established that glutathione plays some part in glucolysis it is at present impossible to say whether it is a coenzyme or not. The results obtained with glutathione are contained in Table 4.

Table 4. *Effect of glutathione*

	mg. lactic acid produced by 5 ml. extract in hr. at 37° from glucose	
Dilution of extracts	With GSH	Without GSH
5 hr. dialysed, 1 : 15 dilut.	7·8	5·1
,, ,,	6·6	4·1
Not dialysed, 1 : 100 dilut.	47·5	41·3
18 hr. dialysed, 1 : 45 dilut.	14·0	10·2
5 hr. dialysed, 1 : 15 dilut.	31·0	21·0
Not dialysed, 1 : 50 dilut.	31·0	24·0
Not dialysed, 1 : 25 dilut.	29·0	18·0
22 hr. dialysed, 1 : 50 dilut.	24·5	18·0

(*f*) *The possible participation of a further coenzyme.* If the extracts are diluted in proportion of 1 part of brain to 250, the activity of the extracts is about $\frac{1}{3}$ of the 1 : 100 dilution. The addition of boiled extract of brain activates these extracts to a rate corresponding to the 1 : 100 dilution. This shows that in addition to the coenzymes described previously, probably a further coenzyme participates in brain glucolysis.

Lactic acid production from various substrates

(*a*) *Hexoses and pentoses.* The rate of lactic acid production in brain extract from various substrates is shown in Table 5. It appears that from glucose, mannose and fructose large amounts of lactic acid are produced. The rate of lactic acid production from fructose is about 10 % higher, from mannose about 10 % lower, than from glucose. Practically no lactic acid is formed from the other sugars.

The most striking fact in these experiments is the large amount of lactic acid produced from fructose. In all the previous experiments made on brain pulp it was found that no lactic acid can be produced from fructose. Needham & Nowiński [1937] have shown that embryonic tissue is also unable to convert

fructose into lactic acid. Nevertheless brain tissue can oxidize fructose within R.Q. of unity, although unable to form lactic acid from it as was shown by Loebel [1925] and by Dickens & Greville [1935]. It is therefore impossible to suppose that fructose cannot enter into the brain cells. The high rate of lactic acid production from fructose by brain extracts is therefore the more surprising. No explanation can be offered for this fact.

In Table 5 are also a few experiments with glucose and glycogen added separately and together to the same extract. They show that the lactic acid formed from glucose is equal in amount to that formed from glucose and glycogen together. The additive behaviour of these two substrates observed by Ashford & Holmes [1929] with brain pulp is not seen in brain extracts. It seems, therefore, that the glucolytic and the glycogenolytic enzyme systems in brain, though probably different, have common components.

Table 5. *Lactic acid formation from different substances in brain extracts*

The lactic acid formation is calculated for 5 ml. of extract corresponding to 1 g. of fresh brain. The values are averages from at least 4 experiments

Substance added	mg. lactic acid formed in 1 hr. at 37°
Glucose	42·1
Glycogen	14·2
Glucose + glycogen	41·0
Fructose	49·5
Mannose	34·2
Galactose	0·9
Arabinose, xylose and sorbose	0
Embden ester	3·1
Robison ester	1·6
Hexosediphosphate	0·6
Phosphoglyceric acid	3·8
α-Glycerophosphate	2·1
Pyruvic acid	1·2
Phosphopyruvic acid	1·3
Hexosediphosphate with 10^{-5} *M* arsenate	1·8
Hexosediphosphate with 10^{-3} *M* arsenate	2·9

(*b*) *Phosphorylated intermediary products of muscle glycolysis.* In Table 5 the extent of lactic acid production by brain extract from phosphorylated intermediary products of muscle glycolysis may be seen. It appears that none of these substances is able to give rise to any substantial amount of lactic acid in brain extract, and the combination of these substances also gave negative results. It must be concluded, therefore, that none of the known phosphorylated intermediary products of muscle glycolysis can be considered as such in brain glycolysis. Experiments made with Hdp in the presence of 10^{-3} *M* arsenate have shown only a very small extent of acceleration of lactic acid formation from Hdp.

The blocking of glycolysis from Hdp and monophosphate occurs probably at the triosephosphate stage. The same was found by Needham & Nowiński [1937] for embryonic tissue. This is evident from the following experiments. A flask, containing 2 ml. of brain extract, Hdp and coenzymes as usual, was incubated at 37°. Lactic acid, inorganic phosphate and triosephosphate were estimated in aliquots at the beginning, after 10 min. and after 60 min. of incubation. 0·78 mg. lactic acid, 5·6 mg. inorganic P and 0·1 mg. alkali-labile P were found at the

beginning; 1·25 mg. lactic acid, 0·62 mg. alkali-labile P and 5·8 mg. inorganic P after 10 min. of incubation; and 1·27 mg. lactic acid, 6·3 mg. inorganic P and 0·66 mg. alkali-labile P at the end of the incubation period. This experiment shows that the equilibrium under our experimental conditions between Hdp and triosephosphate is established after about 10 min. at 37° and that from this time on the lactic acid and the triosephosphate contents remain unchanged. It is evident, therefore, that no lactic acid is formed from Hdp by brain extracts and that the increase in lactic acid appearing after 10 min. of incubation is due to the artificial transformation during the analysis of triosephosphate into lactic acid by the alkaline copper-lime solution. In order to avoid errors it is advisable to estimate the initial value of lactic acid, in experiments in which Hdp is present, after 10 min. of incubation at 37°, i.e. at a time when the equilibrium between Hdp and triosephosphate is established.

Inhibition of glucolysis in brain extracts by cytolysed brain pulp

In a previous paper [Geiger, 1939] the inhibiting effect of cytolysed brain on muscle glycolysis and on yeast fermentation was described. This inhibitor being inactive towards intact cells, its action on brain glycolysis could not be tested. It is now found that cytolysed brain tissue inhibits glycolysis in brain extracts. In the experiments in Fig. 4 various amounts of washed cytolysed brain were added to 1 ml. of brain extract, and the whole was made up to 10 ml. with the usual additions.

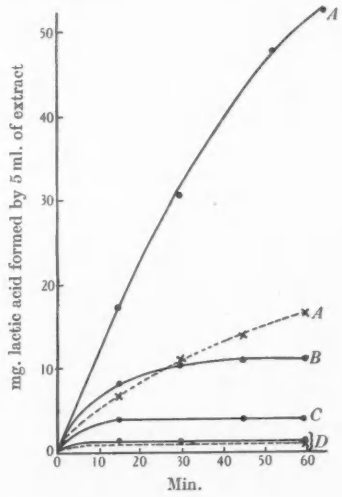


Fig. 4.

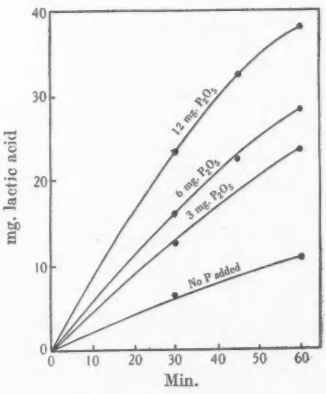


Fig. 5.

Fig. 4. The inhibiting effect of cytolysed brain pulp on the glycolysis of active brain extract. —— glucose, - - - - glycogen. *A*, brain extract alone; *B*, 0·2 g. cytolysed brain added; *C*, 0·3 g. cytolysed brain added; *D*, 0·4 g. cytolysed brain added to a total volume of 10 ml. containing 1 ml. of brain extract.

Fig. 5. The effect of the addition of different amounts of P to 10 ml. of brain extract. Lactic acid is calculated for 1 g. of fresh brain.

The role of phosphorus in glycolysis of brain extracts

(*a*) *The necessity for inorganic phosphorus.* Up to the present brain glucolysis was considered to be mainly a non-phosphorylating glucolysis. Ashford [1934] was unable to obtain any phosphorylation with glycolysing brain slices and with brei. Ashford & Holmes [1929] have also shown that in brain glucolysis the presence of inorganic P is unnecessary. The same conclusions were reached recently by us in experiments on brain pulp in isotonic solution. Euler *et al.* [1936], however, observed the disappearance of inorganic P in brain extracts of low glycolytic activity.

The present experiment shows that in contrast to the results obtained on brain tissue, glycolysis in brain extracts is dependent on the presence of inorganic phosphate. In Fig. 5 experiments are shown in which 0·5 ml. of fresh brain extract was made up to 10 ml. with the usual additions and incubated at 37°. One experiment with glucose and one with glycogen are shown in Fig. 5. The same experiment was repeated with dialysed brain extracts. The results show that there is no glycolysis at all in the absence of inorganic phosphate. The experiments with dialysed extracts are contained in Table 6. With respect to the need of inorganic phosphates, brain extract seems to differ from brain tissue.

Table 6. *The effect of inorganic phosphate on glucolysis in dialysed brain extracts*

The extracts were dialysed for 25 hr. against 0·6 % NaCl solution. 2 ml. of extract were made up to 10 ml. with the usual additions

Phosphate added (as mg. P_2O_5)	mg. lactic acid formed by 5 ml. extract in 1 hr. at 37°
None	0·21
3	5·66
6	9·60
12	13·30
20	13·50

(*b*) *Phosphoric ester formation in brain extracts.* In a second series of experiments the question if phosphorus is esterified during glycolysis was examined. The conditions in these experiments varied with regard to the dilution of the extracts. In the experiments contained in Fig. 6, 1 ml. of brain extract was diluted with the usual additions to 3·7 ml. and incubated at 37°.

It is evident that simultaneously with lactic acid formation from glucose, fructose and mannose, inorganic phosphate disappears. No esterification of phosphate was observed during lactic acid formation from glycogen under these conditions. The esterification of phosphate runs parallel with the formation of lactic acid and ceases together with the latter. As it was seen that phosphorylation is more marked in concentrated extracts than in diluted ones, these experiments were made on concentrated extracts. On the other hand water extracts had to be used. Thus, the rates of lactic acid formation diminish after about 30 min. incubation at 37°. This drop of the glycolytic rate was due mainly to the glycolytic inhibitor present in the brain extracts.

The phosphoric ester formation was well marked in every experiment. It appears, however, that the amount of phosphoric ester found is influenced by the dilution of the extracts. In several experiments with different dilutions of the same brain extract more phosphoric ester was found with the less diluted extract

with identical amounts of lactic acid. In these experiments, which are contained in Table 7, 1 ml. of brain extract was diluted to 3 and to 10 ml. Fructose and glucose were used as substrates.

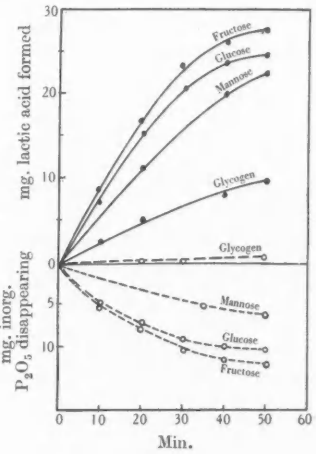


Fig. 6.

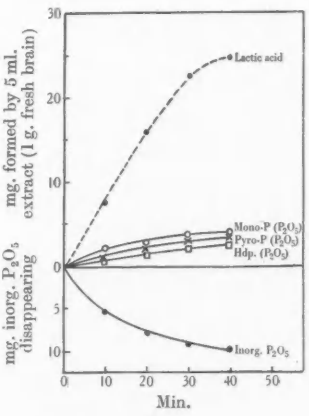


Fig. 7.

Fig. 6. The disappearance of inorganic P in brain extract during lactic acid formation from different substrates. The dotted lines show the changes in inorganic P, the continuous ones the lactic acid produced by extract from 1 g. of fresh brain.

Fig. 7. The distribution of the P compounds formed during glucolysis. In this experiment the substance was glucose.

Table 7. *The effect of dilution of the extracts on phosphoric ester formation*

mg. lactic acid formed and phosphorus esterified, respectively, by 5 ml. of brain extract

Dilution of the extract	1 : 3	1 : 10	1 : 3	1 : 10
Substrate	Glucose		Fructose	
In 30 min.	21·2, 11·7	25·9, 4·0	23·3, 12·0	28·7, 6·22
In 60 min.	24·4, 12·7	45·0, 6·2	25·0, 13·5	49·2, 6·95

It was rather surprising to find no phosphoric ester formation with glycogen. It was thought possible that in this case the formation of phosphoric esters is a slower process than the dephosphorylation. It was tried, therefore, to alter the relative rates of these two processes by changing the temperature. In experiments with glycogen at 20° a small but regular disappearance of inorganic P was seen. The lactic acid formation at this temperature was also very small. One of these experiments is given in Table 8. By comparing the relative amounts of phosphoric ester to lactic acid formed at different temperatures, we find that relatively larger amounts of phosphoric esters accumulate at lower temperature. The lack of phosphorylation of glycogen at 37° must be therefore only apparent, the low rate of phosphorylation and the relatively high rate of dephosphorylation masking the actual esterification. It is possible that the apparent lack of phosphorylation in intact brain cells may also be explained in the same way.

Table 8. *The phosphorylation of glucose and of glycogen at different temperatures*

	Glucose				Glycogen			
	At 37°		At 22°		At 37°		At 22°	
	mg. lactic acid formed	mg. P_2O_5 esterified	mg. lactic acid formed	mg. P_2O_5 esterified	mg. lactic acid formed	mg. P_2O_5 esterified	mg. lactic acid formed	mg. P_2O_5 esterified
In 30 min.	12·1	—	2·9	2·0	3·6	0	1·2	0·5
In 60 min.	21·4	7·7	5·9	3·8	7·1	0	2·3	0·9
In 90 min.	—	—	8·8	5·4	9·9	0	3·2	1·4

(*c*) *The nature of P-esters formed from glucose and from fructose.* As in the present paper it is not intended to deal in particular with the phosphorylation cycles, no attempt was made to isolate and to identify the P-esters formed during glucolysis: this will be the subject of a following paper. For the present it was attempted merely to obtain an idea of the nature of these phosphoric esters as far as is possible by the usual indirect methods such as acid hydrolysis and the solubility of the Ba salts. In a series of experiments the time relationship between the formation of the different P-esters and of lactic acid was examined. Glucose and fructose gave identical results in this respect. All the experiments were made on two different dilutions of brain extracts. In the experiments contained in Fig. 7, 2 ml. of brain extract were made up with the usual additions to 8 ml. The figures are calculated for 5 ml. of extract.

The average figure from 14 experiments shows that about 24% of the disappeared inorganic P are converted into the pyrophosphate fraction (max. 37%, min. 18%), 21% into Hdp, 6% into phosphopyruvic acid and about 45–50% into a phosphoric ester which is not hydrolysed in 3 hr. in *N* acid. For brevity's sake single experiments are omitted.

It was ascertained that the pyrophosphate fraction did not include CE. For this purpose the trichloroacetic acid filtrate was made just alkaline to phenolphthalein and an excess of Ba-acetate was added. The filtrate was treated according to Cori. On the first Ba-precipitate as well as on the alcohol precipitate acid hydrolysis was carried out according to Lohmann. The results show that about 95% of the easily hydrolysable fraction is pyrophosphate. About 21% of the difficultly hydrolysable fraction (not hydrolysed in 3 hr.) was precipitable by Ba at *p*H 8·5; the other 75% was precipitated at the same *p*H after the addition of 2 volumes of alcohol.

(*d*) *Effect of glycolysis inhibitors on phosphorylation.* The addition of fluoride to glycolysing brain extracts causes the accumulation of phosphoglyceric acid as in muscle extracts.

In the presence of *M*/40 fluoride no lactic acid was formed and only a very small amount of inorganic phosphate was esterified. On adding large amounts of Ap, however, a larger amount of a difficultly hydrolysable ester, presumably phosphoglyceric acid, accumulated. *M*/100 fluoride caused a 96% inhibition of lactic acid formation. One experiment of this type is reported in Table 9.

In the following experiments the effect of pyruvic acid was investigated in the presence of fluoride and of glucose. In all these experiments large amounts of Ap were present. Under these conditions lactic acid is formed and P is transformed into a difficultly hydrolysable ester, presumed to be phosphoglyceric acid.

Table 9. *The effect of fluoride on brain extracts*

Substrate	Glucose	Glucose	Glucose	Glycogen
Fluoride conc.	0·3 *M*	0·3 *M*	0·3 *M*	0·3 *M*
mg. Ap P_2O_5 added	1·70	0·78	0·14	1·88
mg. inorg. P_2O_5 esterified	2·72	1·02	0·08	2·12
mg. P_2O_5 transformed into difficultly hydrolysable ester	4·02	1·41	0·11	3·24
mg. Ap P_2O_5 found at the end of exp.	0·41	0·18	—	0·53
Duration of exp. (min.)	30	30	30	30

The lactic acid formation from glucose is inhibited to about 50 % by the addition of 0·03 *M* pyruvate. The same concentration of pyruvate, however, did not inhibit the lactic acid formation from glycogen. The pyruvic acid used in these experiments was redistilled in vacuum from the commercial product. No lactic acid was formed and no phosphates were esterified with pyruvate and with fluoride in the absence of glucose or glycogen. These facts are illustrated in Figs. 8 and 9.

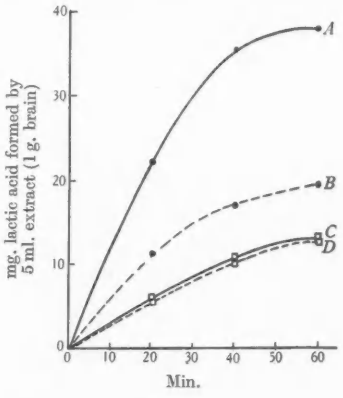


Fig. 8.

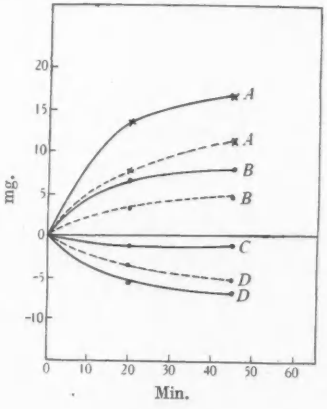


Fig. 9.

Fig. 8. The effect of pyruvic acid (0·03 *M* Na pyruvate) on lactic acid formation from glucose and from glycogen. *A*, glucose; *B*, glucose and pyruvate; *C*, glycogen; *D*, glycogen and pyruvate.

Fig. 9. Formation of lactic acid and of phosphoglyceric acid in the presence of *M*/40 fluoride and of pyruvic acid. From —— glucose and from - - - - - glycogen. *A*, lactic acid formed; *B*, phosphoglyceric acid formed; *C*, Ap disappeared; *D*, inorganic P disappeared. The amounts are calculated for 5 ml. of extract corresponding to 1 g. of fresh brain.

The experiments described here suggest a similar course of esterification in brain extract to one of those found by Meyerhof & Kiessling [1935, 1, 2] in muscle extract. It is impossible, however, to adopt at present any scheme which includes the intermediary formation of Hdp, or of triosephosphate, as brain extract does not transform either of these substances into lactic acid.

The effect of iodoacetate. The accumulation of Hdp from added glucose can be demonstrated in brain extracts in the presence of 0·0002 *M* iodoacetate and of large amounts of Ap. These experiments show that about 50 % of the added Ap is converted into a phosphoric ester which is completely hydrolysed by *N* acid in 180 min. at 100°: it is probably Hdp. At the same time an equal amount

of the Ap-phosphorus is split off. No lactic acid was formed under these conditions. One experiment of this type is contained in Table 10.

Table 10. *The accumulation of hexosediphosphate in the presence of 0·0002* M *iodoacetate*

5 ml. of brain extract

	At start mg.	After 30 min. incub. at 37° mg.
Inorganic P_2O_5	11·32	14·36
Ap.-P_2O_5	7·78	1·24
Triose-P_2O_5	0·18	0·74
Hexosediphosphate-P_2O_5	0·29	2·81
Difficultly hydrolys.-P_2O_5	0·31	0·95

It is evident from this experiment that brain extract may form Hdp from glucose. On the other hand, however, it was shown that Hdp cannot be converted into lactic acid by brain extract. It cannot be concluded therefore from this experiment that Hdp plays any significant role in brain glycolysis. The relatively very slow transformation of Hdp into triosephosphate in brain seems to indicate also that Hdp is not connected with the main route of glucose breakdown.

Pyruvic acid

In several experiments attempts were made to demonstrate the formation of pyruvic acid. In most of these the trichloroacetic acid filtrate of brain extract was precipitated with 2:4-dinitrophenylhydrazine. In other experiments the trichloroacetic acid filtrate was titrated with bisulphite. In no experiment was it possible to obtain positive evidence of pyruvic acid formation. On the other hand the formation of small amounts of phosphopyruvic acid could be demonstrated by estimating this substance according to Meyerhof & Kiessling [1935, 1]. Methylglyoxal formation could not be observed under the present experimental conditions.

The effect of several factors on the rate of glycolysis in brain extracts

The *p*H-optimum for glycolysis in brain extracts is 7–7·5. For obtaining the maximum rate the addition of small amounts of K is also necessary. On the

Table 11. *The effect of several factors on glycolytic rates in brain extracts*

In these experiments 1 ml. of 1 : 5 water-extract of brain was diluted to 10 ml. with the usual additions. The resulting lactic acid production is calculated for amounts of extracts corresponding to 1 ml. of fresh brain. Glucose was used as substrate.

	mg. lactic acid produced in 1 hr. at 37°
The effect of Ca	
Without Ca	51·6
With 0·005 *M* Ca	47·2
With 0·01 *M* Ca	20·5
The effect of *p*H	
*p*H 6·0	39·7
*p*H 6·5	44·0
*p*H 7·0	48·4
*p*H 7·5	33·3
*p*H 8·0	26·2
The effect of O_2 and of K	
In Ringer-solution (anaerobic)	48·1
In Ringer-solution (in O_2)	34·0
In 0·1 *M* KCl solution (anaerobic)	25·6
In 0·1 *M* KCl solution (in O_2)	18·0

other hand Ca and Na inhibit glycolysis, Ca doing so even in very low concentrations.

Aerobic experiments have shown that O_2 inhibits glucolysis in the extracts by about 30–40 %. The addition of KCl in concentrations up to 0·1 *M* did not change this behaviour, but the glycolytic rates in the presence of such amounts of K were very low.

Experiments made at different temperatures show the dependence of the glycolytic rate upon temperature. It seems however that the temperature factor in the case of phosphorylation is smaller than that in glycolysis.

Some of the experiments mentioned under this heading are contained in Table 11.

Discussion

The preparation of glycolytically active brain extracts depends mainly on two factors. The first is the presence of a certain concentration of Mg, the second a relatively high dilution of the extract. The presence of a glycolysis inhibitor in the extracts becomes apparent very soon if concentrated extracts are used. By observing these two conditions it is easy to prepare brain extracts with high and constant glycolytic rate.

Although it is not yet possible to advance any hypothesis concerning the path of glucose breakdown in brain, it is evident that several significant differences exist between the glycolytic processes in brain and in muscle.

The glycolytic substrates in brain are different from those in muscle. In brain extracts practically no lactic acid is formed from any of the phosphorylated intermediary products of muscle glycolysis. The rate of lactic acid formation from glycogen in brain extracts is about a third of that of glucose. In addition to the known coenzymes of muscle glycolysis, CrP and possibly also glutathione have coenzymic functions in brain glycolysis. The optimum concentration of Mg in brain is about 3 times as high as in muscle.

On the other hand, the formation of phosphoric esters during glycolysis is a phenomenon common to brain and to muscle extracts. In the presence of glucose, fluoride and of pyruvic acid there is an accumulation of phosphoglyceric acid in brain extracts as well as in muscle extracts. In the presence of iodoacetate and of large amounts of Ap the accumulation of Hdp can be observed in brain extracts. It must be admitted that the evidence presented here is somewhat contradictory. It is difficult to explain the failure of brain extract to form lactic acid from such substances, which are formed by the extract from glucose. To explain these discrepancies it may be assumed that the phosphorylated products which are found in the brain extract in the presence of fluoride or iodoacetate do not represent intermediary products of glucolysis, but only a by-way. On the other hand, nothing is yet known about the nature of the phosphorus compounds which are formed during glucolysis in brain extracts. It seems that they consist mainly of compounds which hydrolyse less readily than Hdp.

The experiments also show that the differences formerly observed between brain and muscle glycolysis are not due to differences in permeability, for they still exist when muscle extracts and brain extracts are compared.

An interesting problem is presented by the peculiar behaviour of fructose in brain tissue and in brain extracts. Lack of permeability cannot be held responsible for the failure of brain tissue to form lactic acid from this sugar, since it is oxidized like glucose. On the other hand brain extract forms lactic acid from fructose at a higher rate than from glucose.

SUMMARY

The preparation is described of brain extracts, the glycolytic activity of which exceeds that of brain slices by about 4 times.

Dialysis against water destroys the activity of extracts irreversibly. By dialysing against 0·6 % NaCl solution the loss of activity on dialysis is avoided.

Magnesium has to be present in a concentration of 0·013 *M* in order to obtain optimum glucolysis. Lower or higher concentrations diminish the rate of glucolysis. Hexosediphosphate causes a small increase of the glycolytic rate if added in minute amounts.

Phosphocreatine has to be added to dialysed brain extracts in order to obtain glycolysis. This substance cannot be replaced by creatine, or by any of the coenzymes. In fresh extracts the addition of phosphocreatine is only necessary when glucose is the substrate: from glycogen lactic acid is formed without the addition of phosphocreatine. In fresh extracts the effect of creatinephosphate is inconstant.

Glutathione activates brain glycolysis, but its effect is subject to marked variations.

Adenosinetriphosphate and cozymase are both coenzymes of brain glycolysis. It is probable that a yet unknown coenzyme also participates in brain glycolysis.

Large amounts of lactic acid are formed from fructose, glucose and mannose. The rate of lactic acid formation is about a third of that of glucose. Little or no lactic acid is formed from other sugars. Practically no lactic acid is formed from any of the phosphorylated intermediary products of muscle glycolysis. In the presence of arsenate very small amounts of lactic acid are formed from hexosediphosphate.

The presence of inorganic phosphate is necessary for glycolysis in brain extracts. Inorganic phosphate is esterified during glycolysis of all the lactic acid-forming carbohydrates.

The inorganic phosphate esterified during glycolysis is converted mainly into a monophosphate, and to a smaller extent into a pyrophosphate and hexosediphosphate. In the presence of fluoride and of pyruvic acid an ester, probably phosphopyruvic acid which is formed from glucose, accumulates. Extracts poisoned with iodoacetate transfer P from adenosinetriphosphate to glucose, forming hexosediphosphate. Small amounts of phosphopyruvic acid are formed during glycolysis.

The effect of several other factors on glycolysis is described.

REFERENCES

Ashford (1934). *Biochem. J.* **28**, 2228.
—— & Holmes (1929). *Biochem. J.* **23**, 748.
Boyland, Boyland & Greville (1937). *Biochem. J.* **31**, 461.
Case & Cook (1931). *Biochem. J.* **25**, 1319.
Cori, Colowick & Cori (1937). *J. biol. Chem.* **121**, 465.
Dickens & Greville (1935). *Biochem. J.* **29**, 1468.
Euler, Günther & Vestin (1936). *Hoppe-Seyl. Z.* **240**, 265.
Geiger (1935). *Biochem. J.* **29**, 811.
—— (1939). *Biochem. J.* **33**, 877.
—— & Magnes (1939). *Biochem. J.* **33**, 866.

Kendall & Stickland (1937). *Biochem. J.* **31**, 1758.
Loebel (1925). *Biochem. Z.* **161**, 219.
Lohmann (1928). *Biochem. Z.* **194**, 306.
Meyerhof & Kiessling (1935, 1). *Biochem. Z.* **281**, 250.
—— —— (1935, 2). *Biochem. Z.* **283**, 83.
—— Schulz & Schuster (1937). *Biochem. Z.* **293**, 309.
Needham & Lehmann (1937). *Biochem. J.* **31**, 1210.
—— & Nowiński (1937). *Biochem. J.* **31**, 1165.
Ohlmeyer (1936). *Biochem. Z.* **287**, 212.
Ostern, Guthke & Trsakovec (1936). *Hoppe-Seyl. Z.* **243**, 9.
Parnas (1937). *Ergebn. Enzymforsch.* **6**, 57.

58. CRYSTALLINE LACTIC DEHYDROGENASE FROM HEART MUSCLE

BY FERENC BRUNO STRAUB

From the Molteno Institute, Cambridge, England, and the Institute of Medical Chemistry, Szeged, Hungary

(*Received 10 January 1940*)

THE reduction of methylene blue by lactic acid, or in other words, the dehydrogenation of lactic acid in the presence of extracts from animal tissues, is now known to be catalysed by a system composed of two enzymes (lactic dehydrogenase and diaphorase flavoprotein) and cozymase [Dewan & Green, 1938; Euler & Hellström, 1938]. The chemical reactions involved in this process may be written [Corran *et al.* 1939]:

(1) Lactic acid + cozymase $\rightleftharpoons$ pyruvic acid + reduced cozymase.

(2) Reduced cozymase + flavoprotein → cozymase + reduced flavoprotein.

(3) Reduced flavoprotein + methylene blue → flavoprotein + leucomethylene blue.

Reactions (2) and (3) have been described in an earlier paper [Corran *et al.* 1939] and it was shown that the flavoprotein which catalyses these reactions can be isolated from animal tissues [Straub, 1939].

Reaction (1) is catalysed by an enzyme, which can be extracted with distilled water from the tissues. The equilibrium of the reversible reaction (1) is very much in favour of lactic acid formation [Euler *et al.* 1937]. Its action is similar to that of alcohol dehydrogenase (acetaldehyde reductase of Negelein & Wulff [1937]), an enzyme, isolated from yeast, which catalyses the reduction of cozymase by ethyl alcohol and the oxidation of reduced cozymase by acetaldehyde. Our enzyme can therefore be considered either as lactic dehydrogenase or as pyruvic reductase.

But its most important function is the part it plays in the lactic fermentation, being the enzyme which catalyses the last step of lactic fermentation, i.e. the reduction of pyruvic acid to lactic acid.

In order to study the kinetics of these enzymic reactions it seemed desirable to isolate the components. In the present paper the isolation of lactic dehydrogenase in crystalline form is described.

Catalytic test

The purity of the enzyme preparations was determined throughout this work by the "lactic test" as devised by Green & Brosteaux [1936]. This test consists in the measurement of the O_2 uptake which occurs when lactic dehydrogenase is added to a mixture of lactic acid, cozymase, methylene blue, strong cyanide and flavoprotein. The purified flavoprotein was prepared from bullocks' hearts according to the method described in an earlier paper [Straub, 1939]. If any one of these ingredients was omitted no O_2 uptake could be detected. The test can be used as a measure of the quantity of lactic dehydrogenase only if care is taken to make the latter the limiting factor. All the other reactants are therefore taken in large excess and only small O_2 uptakes are measured, e.g. when using

the amounts of ingredients given in Table 1, the O_2 uptake should not exceed 40 μl. per 10 min. Below this value, the rate of O_2 uptake is proportional to the amount of lactic dehydrogenase added.

The Q_{O_2} which can be calculated from the data given in Table 1 cannot be regarded as an absolute measure of the enzyme activity under normal conditions for various reasons, amongst which the most important is the presence of strong cyanide. A detailed study of the activity of the enzyme under more physiological conditions will follow in another paper.

Preparation of the enzyme

(1) *Extraction and adsorption on Ca-phosphate.* Four bullocks' hearts are freed from fat and minced through an ordinary mincer. 5·5 kg. of the mince are mixed with 16·5 l. of ice-cold distilled water and gently stirred for 15–20 min. The fluid is separated from the muscle particles by squeezing through a thin cloth. The water extract, which contains the enzyme, is now mixed with 3 l. of a $Ca_3(PO_4)_2$ gel (freshly prepared, neutralized and washed with tap and distilled water 5–6 times). The amount of Ca-phosphate added is 5 g. for each l. of extract, i.e. 82 g. in the present case. After mixing thoroughly, the Ca-phosphate, which contains the enzyme, is centrifuged off in a Sharples centrifuge. From this gel the enzyme is eluted by mixing it with 3·5 l. of *M*/5 phosphate buffer *p*H 7·2. The Ca-phosphate is centrifuged off and discarded.

(2) *First fractionation with* $(NH_4)_2SO_4$. To the eluate (3·2 l.), after it has cooled down to 10°, 1250 g. of $(NH_4)_2SO_4$ are added (0·6 saturated). The precipitate is filtered off through fluted filters in the cold (4–6 hr.). The clear red filtrate is discarded and the precipitate dissolved in 400 ml. of *M*/10 *p*H 7·2 phosphate buffer, giving 480 ml. of a brownish solution of the enzyme.

(3) *First precipitation with acetone at 13°.* To 480 ml. of this solution, cooled to 0°, 290 ml. of very cold (−15°) acetone are added. The temperature rises to +13° and the mixture is kept at this temperature for 10 min. The precipitate is centrifuged down in cooled tubes and the fluids (two layers) are discarded.

(4) *Second precipitation with* $(NH_4)_2SO_4$. The precipitate is suspended in 200 ml. of 0·3 saturated ammonium sulphate solution, when the great bulk of denatured protein does not redissolve. The undissolved part is centrifuged off in cooled tubes at 0°. From the solution, which contains the enzyme, the acetone is removed by vacuum distillation. The sulphate content of the 190 ml. of solution is 0·32 saturated. A further 21·6 g. of ammonium sulphate are dissolved in it (0·5 saturated). The precipitate which is formed contains the enzyme and it is filtered off through a fluted filter. On dissolving the precipitate in distilled water, 55 ml. of a slightly yellow solution are obtained.

(5) *Second precipitation with acetone at 18°.* After this solution has been cooled to 0°, 33 ml. of acetone at −15° are added. After 10 min. at 18° the precipitate is centrifuged off at 0° and redissolved in 25 ml. 0·3 saturated ammonium sulphate solution. Without removing the insoluble proteins, the mixture is dialysed against 1 l. of 0·3 saturated ammonium sulphate at 0° for 12 hr. It is then centrifuged, giving 22 ml. of a clear, almost colourless solution.

(6) *Crystallization.* By the addition of 1·32 g. $(NH_4)_2SO_4$ to the 22 ml. of solution obtained in the previous step, the salt concentration is brought to 0·4 saturation and the precipitate is rapidly centrifuged off. To the clear supernatant fluid a saturated solution of ammonium sulphate is added drop by drop. When the concentration of ammonium sulphate reaches 0·5 saturation, the crystallization is complete. The precipitate is centrifuged off at 10,000 r.p.m.

LINDA HALL LIBRARY
KANSAS CITY, MO.

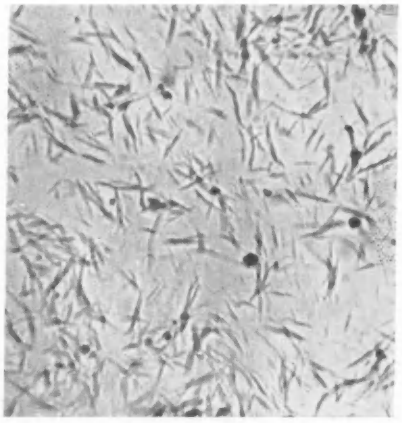


Fig. 1.

Fig. 2.

Crystalline lactic dehydrogenase. ×540.

Table 1. *Dehydrogenation of lactic acid, catalysed by different lactic dehydrogenase preparations*

In each manometer vessel are present: 0·9 ml. of a 0·4% methylene blue solution, 0·3 ml. of 10% Na lactate, 0·2 mg. muscle flavoprotein, 0·2 ml. of a freshly prepared, neutralized $M/2$ KCN solution, 2·5 mg. of cozymase preparation (about 20% pure) and phosphate buffer pH 7·2 giving a final concentration of $M/20$. Total volume 4 ml.

Temperature: 38°. Gas space: air. Warburg manometers

Lactic dehydrogenase added to the above mixture	O_2 uptake (μl.) In 10 min.	In 20 min.
None	0	0
Water extract of heart muscle mg. protein		
0·148	25·4	52·5
0·222	38·3	69·0
Crystalline enzyme mg. protein		
0·0024	24·0	47·4
0·0036	35·5	66·8

The crystals are very fine needles, which tend to form clusters. Their refractive index differs so slightly from that of the ammonium sulphate solution that it is extremely difficult to get a good photograph of them. If a drop of methylene blue solution is added, the crystals take up the dye very strongly, remaining unchanged in form. Fig. 1 shows the crystals themselves and Fig. 2 shows them when stained with methylene blue.

The crystals dissolve readily in water, giving a perfectly clear and colourless solution. Recrystallization does not change their activity and the form and size of the crystals remain the same after three recrystallizations.

If a solution of the crystalline enzyme is dialysed until free from salts, the enzyme is inactivated and it is precipitated as a denatured protein, which will not dissolve again even in the presence of salts. However, if kept in 0·1 saturated ammonium sulphate solution in the cold, the activity of the pure enzyme does not change in 6 weeks.

Table 2. *Yield of enzyme at various stages of purification*

	Total protein g.	Lactic dehydrogenase g.
Water extract of 5·5 kg. muscle	177	3·5
Ca-phosphate elution	26	2·1
First $(NH_4)_2SO_4$ precipitate	5·2	1·3
First acetone precipitation	2·5	0·9
Second $(NH_4)_2SO_4$ precipitate	1·1	0·65
Second acetone precipitation	—	0·46
Crystals	0·36	0·36

Non-identity of lactic and malic dehydrogenases

The lactic and malic dehydrogenases of animal tissues have been generally regarded as different enzymes [Green & Brosteaux, 1936; Green, 1936]. Das [1937] has brought forward some experimental data suggesting a possible identity of lactic and malic dehydrogenases. It is clear from the complexity of the dehydrogenating mechanism that no conclusive evidence could be expected from the study of the impure preparations.

If crystalline lactic dehydrogenase is used in the test which has been described above (for details see Table 1), even 240 μg. enzyme give no oxidation at all if

lactic acid is replaced by *l*-malic acid, whereas 2·4 μg. enzyme give a good O_2 uptake with lactic acid. If an aqueous extract of heart muscle is added to the test in place of the pure lactic dehydrogenase, then both acids are oxidized, although lactic acid is oxidized much better than *l*-malic acid.

SUMMARY

The enzyme which catalyses the reduction of cozymase by lactic acid and the oxidation of reduced cozymase by pyruvic acid has been prepared in the crystalline form. This enzyme, together with the heart flavoprotein and cozymase, forms a complete system oxidizing lactic acid. Lactic and malic dehydrogenases are not identical.

I am grateful to Prof. D. Keilin and Prof. A. Szent-Györgyi for their advice and interest. The work was done partly (in Szeged) with the help of the Josiah Macy Jr. Foundation, New York.

REFERENCES

Corran, Green & Straub (1939). *Biochem. J.* **33**, 793.
Das (1937). *Biochem. J.* **31**, 1116.
Dewan & Green (1938). *Biochem. J.* **32**, 626.
Euler, Adler, Günther & Hellström (1937). *Hoppe-Seyl. Z.* **245**, 217.
—— & Hellström (1938). *Hoppe-Seyl. Z.* **252**, 31.
Green (1936). *Biochem. J.* **30**, 2095.
—— & Brosteaux (1936). *Biochem. J.* **30**, 1489.
Negelein & Wulff (1937). *Biochem. Z.* **289**, 436.
Straub (1939). *Biochem. J.* **33**, 787.

59. IMMUNOLOGICAL DIFFERENCES OF CRYSTALLINE BENCE-JONES PROTEINS

By LUDVIG HEKTOEN and WILLIAM H. WELKER

From the John McCormick Institute for Infectious Diseases and the Department of Physiological Chemistry, University of Illinois College of Medicine, Chicago, Illinois

(*Received 15 January 1940*)

One of the first suggestions that the protein appearing in the urine that gives the peculiar Bence-Jones reaction might not necessarily be the same protein in all cases was made by Ville & Derrieu [1907]. Later Hopkins & Savory [1911], on the basis of analyses of the amino-acid composition of the proteins from two cases, concluded that they were identical in chemical constitution. Bayne-Jones & Wilson [1922], in a study of the immunological properties of Bence-Jones proteins by means of the precipitin reaction and the anaphylactic reaction, concluded that there were at least two and possibly three groups of Bence-Jones proteins recognizable by the tests used. In their study they used proteins from 5 cases, three diagnosed as multiple myeloma, one carcinoma with metastases to the bones and one without demonstrable lesions in the bones. Only one of the proteins used was reasonably pure. This protein crystallized spontaneously from the urine and was further purified by recrystallization. It could be argued that the differences observed might have been due to the impurities of all but one of the protein preparations used. Robinson [1927] studied the antigenic properties of four preparations of Bence-Jones protein from four cases of myelomatosis by means of the precipitin reaction and came to the conclusion that at least two of these protein preparations were different. These preparations were crude and the comments on the work of Bayne-Jones & Wilson [1922] are applicable here also.

From our previous work on Bence-Jones protein we had available four crystalline proteins from four different patients. We also had some of the crystallized protein furnished us by Dr D. Wright Wilson [1923] and some of the first crystallized protein ever isolated from urine prepared by Noel Paton [1892]. Antisera were prepared for our own four crystalline proteins. Table 1 shows the results of the titration of the six crystalline proteins against these antisera.

Table 1. *Precipitin reactions with the antisera for the crystalline proteins*

	Antisera			
	Bence-Jones 1	Hogan	Mahle	Nelson
Bence-Jones 1 [Hektoen & Welker, 1924]	4	0	0	0
Hogan [Hektoen *et al.* 1924]	0	4+	3	3
Mahle *et al.* [1938]	0	4	4	3
Nelson [Mahle *et al.* 1938]	0	4	3	3
Wilson [1923]	0	3	3	3
Paton [1892]	0	2		

0 = No reaction.
1 = Titre in dilution 1:1000.
2 = Titre in dilution 1:10,000.
3 = Titre in dilution 1:100,000.
4 = Titre in dilution 1:1,000,000.

These results show that the crystalline proteins fall into two distinct immunological groups. It is striking that five out of the six belong to the same group. This is probably due to the fact that the larger group can under certain conditions be obtained readily in crystalline form, while the other group can only be obtained in crystalline form with great difficulty.

From our previous work we had also available non-crystalline Bence-Jones proteins from five other cases. Dr Wilson furnished us with Bence-Jones from five patients. Dr H. O. Calvery & Dr R. H. Freyberg [1935] furnished us with one Bence-Jones protein which they had analysed for the amino-acid composition. Dr Grace Medes [Berglund & Medes, 1935] furnished us with Bence-Jones protein from five patients which she had analysed for their amino-acid composition. Table 2 shows the results of the titration of solutions of the non-crystalline proteins against the antisera for our crystalline proteins.

Table 2. *Precipitin reactions with non-crystalline Bence-Jones proteins*

	Antisera			
	Bence-Jones 1	Hogan	Mahle	Nelson
Wilson:				
1–M.	2	0	±	0
2–T.	1	0	0	0
4–S.	±	0	0	0
3–R.	0	3	3	3
5–2	3	1	2	1
Medes:				
G.G.	3	0	0	0
C.K.	3	0	0	0
A.B.	1	0	0	0
A.G.	0	3	4	3
M.G.	0	3	2	3
Calvery-Freyberg:	0	3	2	3
Hektoen-Welker:				
1–R.	0	3	3	3
2–F.	0	3	3	3
3–S	3	0	0	0
4–D.R.	3	0	0	0
5–M.C.	3	3	4	3

From these results it is clear that these Bence-Jones proteins fall primarily into two main groups. In two of the cases it appears that the patients were eliminating both types (5–2 and 5 M.C.). Some of the samples were relatively insoluble and the low titres with some of the antigens may have been due to the small amount that went into solution. An antiserum was prepared against protein A.B. and its reactions with our antigens placed it into the Bence-Jones 1 group.

The analyses for nitrogen distribution made by Medes on the samples furnished us showed that all of them with the exception of C.K. gave approximately the same results, while C.K. showed marked differences. The results in Table 2 show that three of these proteins belong to Bence-Jones 1 group, among them C.K., and two to group 2. These results are difficult to reconcile unless we assume that not all of a given protein molecule is involved in its antigenic properties and that the chemical configuration of the portion of the molecule involved in its antigenicity determines the character of its antigenicity. The analytical results obtained by Calvery & Freyberg on the protein they isolated differ markedly from those obtained by Medes. The immunological properties of this protein indicate that it belongs to group 2. This result further emphasizes that differences in composition do not necessarily mean different antigenicity.

SUMMARY

With antisera for four different crystalline Bence-Jones proteins, two groups of these proteins have been demonstrated as immunologically distinct. This observation confirms the work of Bayne-Jones & Wilson [1922] and that of Robinson [1927].

Chemical composition as determined by the percentages of the various amino-acids present in the protein is not the sole determining factor of its antigenic properties.

An individual may eliminate both types of these immunologically distinct Bence-Jones proteins at the same time as indicated by two of the cases in this study.

REFERENCES

Bayne-Jones & Wilson (1922). *Johns Hopk. Hosp. Bull.* **32**, 119.
Berglund & Medes (1935). The Kidney in Health and Disease. Philadelphia.
Calvery & Freyberg (1935). *J. biol. Chem.* **109**, 739.
Hektoen, Kretchmer & Welker (1924). *J. Amer. med. Ass.* **83**, 1154.
—— & Welker (1924). *J. infect. Dis.* **34**, 440.
Hopkins & Savory (1911). *J. Physiol.* **42**, 189.
Mahle, Seed & Welker (1938). *Arch. Path.* **26**, 441.
Paton (1892). *Proc. roy. Soc. Edinb.* **19**, 102.
Robinson (1927). *Brit. J. exp. Path.* **8**, 454.
Ville & Derrieu (1907). *Montpellier Med. J.* **24**, 387.
Wilson (1923). *J. biol. Chem.* **56**, 203.

60. THE URINARY EXCRETION OF MANDELIC ACID ADMINISTERED INTRAVENOUSLY TO CATS

BY ROBERT CAMPBELL GARRY AND ISOBEL AGNES SMITH

From the Departments of Physiology and Chemistry, University College, Dundee, University of St Andrews

(*Received 29 January 1940*)

MANDELIC acid, when administered to animals, may be recovered almost quantitatively from the urine. Moreover, when mandelic acid appears in the body as the result of metabolic processes, it is supposed to be excreted without further change [Knoop, 1905; Dakin, 1922; Quick, 1928].

Neubauer [1909], Neubauer & Fischer [1910], Neubauer & Gross [1910] and Neubauer & Warburg [1910] claimed that after giving *r*-phenylaminoacetic acid to dogs by mouth and perfusing dog livers, the following substances could be recovered from the urine and perfusate:

(−)$C_6H_5.CH(NH_2).COOH$.
(−)$C_6H_5.CH(OH).COOH$ (examined after crystallization from benzene).
$C_6H_5.CO.COOH$.
$C_6H_5CO.NHCH_2.COOH$ (presumably from $C_6H_5.COOH$).

When, however, (−)phenylaminoacetic acid was given to dogs in similar fashion it appeared in the urine largely unchanged, while (+)phenylaminoacetic acid was metabolized with relative ease. Both benzoylformic acid and (−)mandelic acid were recovered from the urine in the latter case. When benzoylformic acid was administered to dogs, (−)mandelic acid appeared in the urine. *r*-Mandelic acid, when given by mouth, was practically all recovered in the urine as such, showing, if anything, a slight dextrorotation, there being a mere trace of benzoylformic acid.

In man the results were the same as in the dog, but in rabbits administration of *r*-phenylaminoacetic acid by mouth gave rise to urinary excretion of optically pure (−)phenylaminoacetic acid, benzoylformic acid, hippuric acid, but no mandelic acid.

Neubauer therefore argued that mandelic acid could not be an intermediary between phenylaminoacetic acid and benzoylformic acid. He also believed that (+)phenylaminoacetic acid, the form attacked in the body, could not yield (−)mandelic acid directly because of stereochemical dissimilarity. This latter conclusion is, however, untenable, since Clough [1918] showed that (+)phenylaminoacetic acid has the same stereochemical configuration as (−)mandelic acid.

Neubauer put forward the following scheme for the metabolism of phenylaminoacetic acid:

$$C_6H_5.CH(NH_2).COOH \xrightarrow[\text{oxidative deamination}]{} C_6H_5.CO.COOH \xrightarrow[\text{asymmetric reduction}]{} (-)C_6H_5.CH(OH).COOH.$$

For many years Neubauer's hypothesis has been accepted without question. Wrede [1938], Scholz [1938] and Montenbruck [1938], however, claim that mandelic acid does undergo appreciable metabolic change both in man and in dogs. They gave *r*-mandelic acid as the ammonium salt by mouth, collected

the urine for 72 hr. and recovered an appreciable amount of (−)mandelic acid and of benzoylformic acid. (−)Mandelic acid was also recovered when the ammonium salt of benzoylformic acid was given in similar fashion. Two further experiments were carried out on man. In one case (−)mandelic acid, in the other (+)mandelic acid was given. In neither case was the optically pure acid used. When the laevo-acid was given, 73% was recovered as such, and 7% as benzoylformic acid, while, with the dextro-acid, only 65% was recovered and 14% appeared as benzoylformic acid. These workers therefore suggest that the dextro-form of mandelic acid is more readily metabolized in the body than the laevo-form.

When we started the present investigation early in 1938 we were unaware of the results of these German workers. We accepted the evidence of the previous literature that mandelic acid is resistant to attack in the body [Garry & Smith, 1938].

With the exception of the perfusion experiments on isolated organs, all the above results were obtained by oral administration of the substances under examination. McKenzie [1902] showed that when optically inactive potassium and sodium β-hydroxybutyrates respectively were injected subcutaneously into dogs a small proportion of the β-butyrates was recovered in the urine, and this product was laevorotatory. This partial resolution cannot be ascribed to the action of bacteria and does not therefore fall under the head of any of Pasteur's methods for resolving externally compensated compounds. We are indebted to Prof. McKenzie for the suggestion that it might be of interest to examine the behaviour of mandelic acid when given parenterally. Would it be possible for the living organism, under these conditions, to resolve *r*-mandelic acid into its antimerides?

Methods

Administration. In order to introduce as few variables as possible we gave the mandelic acid intravenously. Decerebrate cats were used to avoid interference from anaesthesia. The urine was collected through a silver cannula tied into the urinary bladder.

To promote an ample flow of urine throughout the experiment, warmed Ringer's fluid, or a 1% solution of NaCl, was slowly run into the external jugular vein. Mandelic acid, as a solution either of the free acid or of the sodium salt, was delivered from a burette into the main stream of fluid entering the jugular vein. The total volume of fluid given varied between 1000 and 1500 ml.; the volume of urine recovered was usually less by several 100 ml.

The urine was collected in small measured amounts, varying from 25 to 100 ml. A few drops of toluene were added to each collecting flask to prevent bacterial action. The *p*H of each sample was determined colorimetrically.

The sodium salt of mandelic acid could be given rapidly in large quantity, for example 10 g. as a 10% solution in 15 min., without distress to the cat. When the free acid was given the administration had to be slow and the solution dilute.

Chemical extraction. The individual samples of urine were evaporated on a water bath to smaller bulk in the presence of $(NH_4)_2SO_4$ and filtered. Thereafter, the filtrate was acidified with H_2SO_4 saturated with $(NH_4)_2SO_4$ and extracted 6 times with ether. Continuous extraction was not used since a large number of individual samples had to be dealt with. The ethereal extracts were dried over anhydrous Na_2SO_4; the bulk of the ether was removed by distillation and the remainder allowed to evaporate at room temperature.

The residues were dried in a vacuum desiccator until constant in weight and then, without further purification, examined polarimetrically at room temperature in acetone using the mercury green line.

Results

Control experiments

Pure optically active (−)mandelic acid has $[\alpha]_{5461}^{20°} - 189{\cdot}9°$ for $c = 5{\cdot}046$ in acetone solution [Roger, 1932].

No lowering of specific rotation was caused when pure (−)mandelic acid was dissolved in water, treated with $(NH_4)_2SO_4$ and extracted in the manner described above. The dextro-acid was similarly unaffected.

When laevo- or dextro-mandelic acid was added to cats' urine in amounts comparable with those recovered during an experiment the rotation of the recovered material was slightly less than the value for the pure forms of the active acid.

Collection of urine from cats during intravenous injection of Ringer's fluid alone showed that the early samples of urine had a small amount of ether-soluble material; this material was optically inactive. Later samples of urine had a negligible amount of extract.

Sodium r-*mandelate*

When the sodium salt of the racemic acid was given there was initially preferential excretion of the laevo-form, the maximum activity being observed in the early samples. Thereafter the material from subsequent samples showed a decreasing laevorotation, usually passing through optical inactivity to dextrorotation (Table 1).

Table 1

4·9 g. *r*-mandelic acid in 1% solution as the sodium salt given intravenously in 554 min.

Time min.	Vol. of urine ml.	Wt. material extracted g.	$[\alpha]_{5461}$	($l = 2$)
118	50	0·32	−31·1°	($c = 1{\cdot}3035$)
155	50	0·18	−12·9°	($c = 0{\cdot}777$)
194	100	0·26	− 5·2°	($c = 1{\cdot}145$)
229	100	0·23	− 2·5°	($c = 1{\cdot}015$)
263	100	0·22	+ 1·6°	($c = 0{\cdot}9315$)
299	100	0·23	+ 3·2°	($c = 0{\cdot}938$)
336	100	0·19	+ 6·5°	($c = 0{\cdot}8425$)
371	100	0·18	+ 7·4°	($c = 0{\cdot}678$)
396	100	0·16	+ 6·6°	($c = 0{\cdot}687$)
423	100	0·16	+ 6·2°	($c = 0{\cdot}643$)
449	100	0·15	+ 5·3°	($c = 0{\cdot}656$)
476	100	0·17	+ 4·9°	($c = 0{\cdot}71$)
501	100	0·21	+ 4·2°	($c = 0{\cdot}9445$)
543	100	0·24	+ 2·8°	($c = 1{\cdot}0545$)
577	100	0·24	+ 4·2°	($c = 1{\cdot}0765$)
608	100	0·19	+ 8·6°	($c = 0{\cdot}756$)

The smaller the amount of salt given in unit time, the more obvious the preferential excretion of the laevo-form of the acid in the early samples of urine (Table 2).

When using the sodium salt of the racemic acid we never obtained a rotation greater than $[\alpha]_{5461} - 65{\cdot}0°$ ($c = 0{\cdot}676$) in acetone, and that was in 0·1 g. of recovered material.

In one case, where 6 g. of *r*-mandelic acid were given in 150 ml. water as the sodium salt, 83 % was recovered in 436 min. In another case 92 % was recovered in 592 min. when 7·5 g. (+)mandelic acid were given.

Table 2

Rapid administration

6 g. *r*-mandelic acid in 4 % solution as the sodium salt given intravenously in 24 min.

Time min.	Vol. of urine ml.	Wt. material extracted g.	$[\alpha]_{5461}$	($l=2$)
15	25	0·23	−18·8°	($c=0·876$)
26	25	0·36	−17·4°	($c=1·462$)
40	25	0·35	−17·2°	($c=1·545$)
52	25	0·29	−15·8°	($c=1·107$)
79	100	0·59	−13·0°	($c=2·3455$)

Slow administration

2 g. *r*-mandelic acid in 1 % solution as the sodium salt given intravenously in 277 min.

Time min.	Vol. of urine ml.	Wt. material extracted g.	$[\alpha]_{5461}$	($l=2$)
104	100	0·21	−55·0°	($c=0·717$)
163	100	0·20	−34·5°	($c=0·884$)
209	100	0·17	−21·8°	($c=0·6645$)
253	100	0·16	−13·7°	($c=0·6215$)
325	125	0·22	−12·3°	($c=0·811$)

Table 3

2 g. *r*-mandelic acid in 1 % solution as the sodium salt given intravenously in two separate lots of 1 g. each.

Time min.	Vol. of urine ml.	Wt. material extracted g.	$[\alpha]_{5461}$	($l=2$)
95	50	0·06	−57·0°	($c=0·395$)
152	50	0·15	−42·9°	($c=0·6405$)
		Administration of acid stopped		
214	100	0·15	−14·7°	($c=0·6115$)
256	100	0·07	+ 6·5°	($c=0·382$)
		Administration of acid resumed		
294	100	0·07	+10·8°	($c=0·277$)
333	100	0·13	− 2·6°	($c=0·576$)
372	100	0·16	− 3·5°	($c=0·71$)
		Administration of acid finally stopped		
416	100	0·19	Inactive	($c=0·7785$)
455	100	0·11	+12·1°	($c=0·4135$)

In several cats administration of the racemic acid was delayed until active diuresis was in progress; the initial preferential excretion of the laevo-form was unaffected. Administration of the acid in two separate quantities, with an interval of 2 hr. between, showed that the optical activity of the excreted material from the first injection followed the usual course. On resumption of the injection of the acid the preferential excretion of the laevo-form became once more apparent (Table 3).

Free r-*mandelic acid*

Administration of free *r*-mandelic acid gave very similar results (Table 4).

Table 4

3·2 g. free *r*-mandelic acid in 1% solution given in 292 min.

Time min.	Vol. of urine ml.	Wt. material extracted g.	$[\alpha]_{5461}$	($l=2$)
76	50	0·07	−82·2°	($c=0·523$)
103	50	0·06	−58·1°	($c=0·516$)
134	100	0·14	−34·2°	($c=0·584$)
165	100	0·14	−26·3°	($c=0·665$)
196	100	0·16	−17·4°	($c=0·631$)
222	100	0·17	−14·7°	($c=0·7485$)
250	100	0·17	− 8·8°	($c=0·7425$)
294	200	0·34	− 6·5°	($c=0·824$)
335	200	0·24	Inactive	($c=0·591$)
382	200	0·16	+ 9·4°	($c=1·067$)
434	200	0·12	+16·9°	($c=0·8$)
503	200	0·15	+19·0°	($c=0·948$)
581	200	0·11	+23·6°	($c=0·954$)

Optically active mandelic acids

We obtained these acids in optically pure form by resolution of the racemic acid with (−)ephedrine [Roger, 1935].

Typical results with the sodium salts of the active acids are shown in Tables 5 and 6, and with the free acids in Tables 7 and 8.

Table 5

7·5 g. (+)mandelic acid in 4% solution as the sodium salt given intravenously in 120 min.

Time min.	Vol. of urine ml.	Wt. material extracted g.	$[\alpha]_{5461}$	($l=2$)
75	100	0·72	+183·9°	($c=2·624$)
95	100	0·71	+186·6°	($c=2·7085$)
116	100	0·83	+185·3°	($c=3·0525$)
176	200	1·51	+184·4°	($c=2·5325$)
248	200	1·10	+181·6°	($c=2·745$)
323	200	0·84	+181·3°	($c=3·086$)
470	300	0·86	+181·6°	($c=3·216$)
587	300	0·34	+170·5°	($c=1·4955$)

Table 6

5 g. (−)mandelic acid in 4% solution as the sodium salt given intravenously in 125 min.

Time min.	Vol. of urine ml.	Wt. material extracted g.	$[\alpha]_{5461}$	($l=2$)
70	100	0·60	−180·7°	($c=2·628$)
98	100	0·56	−183·0°	($c=2·47$)
145	200	0·90	−179·7°	($c=2·5345$)
215	300	0·87	−180·9°	($c=2·501$)
296	300	0·60	−176·7°	($c=2·575$)
420	400	0·54	−172·5°	($c=2·5415$)

Table 7

3·7 g. free (+)mandelic acid in 1% solution given in 246 min.

Time min.	Vol. of urine ml.	Wt. material extracted g.	$[\alpha]_{5461}$	($l=2$)
38	50	0·09	+169·4°	($c=0·121$)
57	50	0·10	+180·8°	($c=0·224$)
72	50	0·08	+185·3°	($c=0·17$)
86	50	0·10	+182·4°	($c=0·2385$)
107	100	0·16	+185·1°	($c=0·543$)
129	100	0·16	+183·6°	($c=0·55$)
171	200	0·36	+186·6°	($c=0·614$)
210	200	0·39	+187·8°	($c=0·724$)
247	120	0·29	+185·3°	($c=1·152$)

Table 8

4·0 g. free (−)mandelic acid in 1% solution given in 295 min.

Time min.	Vol. of urine ml.	Wt. material extracted g.	$[\alpha]_{5461}$	($l=2$)
38	50	0·08	−157·7°	($c=0·333$)
62	50	0·13	−165·7°	($c=0·67$)
93	100	0·25	−178·8°	($c=0·285$)
148	200	0·45	−180·0°	($c=0·715$)
293	400	0·89	−181·9°	($c=1·2815$)
360	200	0·32	−178·7°	($c=1·1925$)

The rotation of the extracted material was usually slightly lower than the full rotation of the injected acid.

Material recovered in the ethereal extract

Droller [1938] found that taking by mouth 4·5 g. "Mandecal", containing roughly 3 g. mandelic acid as calcium mandelate, lowered the *p*H of his urine to 5, and he states that free mandelic acid was present. In our cats, however, when the sodium salt was used the *p*H of the urine ranged from 6·7 to 7·4, and with the free acid the *p*H of the urine never fell below 6·2. It would appear that the cat is able to excrete most of the mandelic acid as a salt. This was borne out by extraction of the urine with ether before and after acidification (Table 9).

Table 9

Material extracted before acidification		Material extracted after acidification	
Wt. (g.)	$[\alpha]_{5461}$	Wt. (g.)	$[\alpha]_{5461}$
Administration of the sodium salt of *r*-mandelic acid			
0·02	—	1·51	−17·1°
0·01	—	0·86	− 4·8°
0·00	—	0·74	− 1·2°
Administration of free *r*-mandelic acid			
0·05	−40·8°	0·34	−56·1°
0·07	−19·4°	0·24	−17·7°
0·07	− 6·3°	0·18	− 5·4°
0·08	+ 6·0°	0·12	+ 6·7°

The material recovered by routine extraction was a dry powder with a slight yellowish colour which was removed completely by boiling the solution of the

acid with charcoal (Norite). Such treatment gave the following results in two cases:

Before decolorization	After decolorization
$[\alpha]_{5461}$ $-58°$ ($c=0{\cdot}548$)	$[\alpha]_{5461}$ $-61°$ ($c=0{\cdot}638$)
$[\alpha]_{5461}$ $-180°$ ($c=2{\cdot}169$)	$[\alpha]_{5461}$ $-184°$ ($c=2{\cdot}281$)

Contamination with benzoylformic acid was looked for by means of the colour reaction with benzene (containing thiophene) and conc. H_2SO_4; there was no sign of the characteristic violet-red colour. Moreover, the extracted material gave no hydrazone when treated with phenylhydrazine in acid solution. Further, when material with a rotation of $-184°$ was dissolved in ether and treated with a saturated solution of sodium bisulphite to trap benzoylformic acid, the rotation was unchanged.

This same sample showed a melting point of 129–132°, with softening at 124°. (−)Mandelic acid melts at 133°, *r*-mandelic acid at 118° and benzoylformic acid at 65–66°.

Three separate samples, A, B and C, of material extracted in routine fashion were submitted to analysis without further purification. A was obtained from an early sample of urine when *r*-mandelic acid was given, B when pure dextro-acid was given and C when pure laevo-acid was given.

	% of		
	C	H	N
Found A	63·2	5·1	Nil
Found B	63·3	5·7	Nil
Found C	62·3	5·5	0·9
Calculated for pure mandelic acid	63·2	5·3	Nil

Equivalent determinations with baryta, using phenolphthalein as indicator, were carried out on three samples of recovered material. One sample from an early stage in an experiment where the racemic acid was given showed marked laevorotation, the second sample was optically inactive, while the third was from a late stage in an experiment and showed dextrorotation. In all three cases the titration values gave equivalents essentially in agreement with the theoretical value for mandelic acid.

Discussion

There is little doubt that the material extracted from the urine by our routine method was in all cases practically entirely mandelic acid. In the experiments of which the results are given in Tables 5–8, the percentages of active mandelic acid in the entire recovered material were, using optical activity as the criterion, 96, 94, 97 and 94 respectively. The assumption that the impurities were optically inactive is supported by the fact that the degree of purity was very similar with both laevo- and dextro-forms.

To obtain such results if the contaminant had been optically active, it must have been of opposite sign in the two contrasting experiments. Thus, the impurity, if derived from mandelic acid, would have to be generated without destruction of the asymmetry of the mandelic acid molecule. On the other hand, if mandelic acid were oxidized partially to benzoylformic acid, and if the latter were to undergo metabolic change back to mandelic acid, the hypothesis of Neubauer would lead us to expect preferential formation of the laevo-form independently of the nature of the mandelic acid originally administered.

The improbability of benzoylformic acid playing any marked part in our experiments extending over a relatively short time is also supported by failure to detect it in the ethereal extract.

There is no support in our results for the hypothesis of Montenbruck [1938] that the dextro-form of mandelic acid is more rapidly metabolized than the laevo-form. We were, however, probably not concerned with metabolic derivatives of mandelic acid to the same extent as other workers, who gave the acid by mouth and collected the urine for several days thereafter. The time allowed for metabolic change and for the excretion of metabolic products was, in our experiments, relatively short.

The use of intravenous administration in our technique eliminated the possibility of bacterial action in the gut and avoided passage through the intestinal mucous membrane. McKenzie & Harden [1903] showed that the laevo-form of mandelic acid is preferentially attacked by micro-organisms.

Actually, within the duration of our experiments there was little evidence for ultimate differential behaviour of the body towards either of the active forms of mandelic acid. Without fractional collection of the urine and optical examination of the excreted fractions, the initial preferential excretion of the laevo-form would have been missed. When the experiment lasted a reasonable length of time the early preferential excretion of the laevo-form was nearly compensated by an excretion of the dextro-form (Fig. 1). In both cases the curves show that

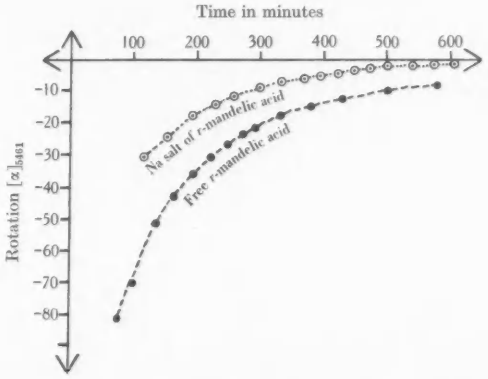


Fig. 1. The curves are drawn from the data recorded in Tables 1 and 4. Each point represents the rotation of the total amount of material excreted up to that time.

the rotation is approaching optical inactivity. Here again, in the cat at least, there is no good evidence to support Montenbruck's hypothesis that the dextro-form is preferentially metabolized in the body when the acid is given intravenously.

There must, however, be an early temporary differential reaction of the cat's body to the two forms of mandelic acid. The preferential excretion of the laevo-form at the outset must leave a preponderance of the dextro-form in the body which finds its expression in the subsequent excess of the dextro-form in the urine. One could postulate preferential metabolic attack on the dextro-form at the outset, but then one would need to invoke the conception of subsequent regeneration of that form to explain the preponderance of this isomer in the later samples of urine. The results from administration of active acids do not support this view.

Henderson & Rule [1939] have shown that percolation of a solution of racemic *p*-phenylenebisiminocamphor through a column of lactose leads to partial resolution due to the dextro-isomeride being more strongly adsorbed on the lactose. Westenbrink [1936] and Westenbrink & Gratama [1937] found that the intestines of pigeons and of frogs absorb the naturally occurring (+)xylose more rapidly than the (−) form. It is conceivable that such phenomena may occur with respect to mandelic acid in the kidney or in some other site in the body.

Still another possible explanation depends on the well-known principle of Marckwald & McKenzie [1899]. These workers showed that when mandelic acid is esterified with (−)menthol, the (−)menthyl (+)mandelate is more rapidly formed than the (−)menthyl (−)mandelate. Moreover, the rate of hydrolysis of (−)menthyl (+)mandelate is greater than that of (−)menthyl (−)mandelate. That such phenomena can occur in the body is strongly suggested by the observations of Dakin [1903, 1, 2; 1905]. He found that, when esters of *r*-mandelic acid are hydrolysed by liver lipase, the rate of hydrolysis of the ester of (+)mandelic acid is greater than the rate of hydrolysis of the ester of (−)mandelic acid. The inference was that the ester of the dextro-form was attacked more rapidly than the ester of the laevo-form. However, when this hypothesis was tested at a later date, Willstätter *et al.* [1928], by taking the dextro- and laevo-esters separately, found that the enzyme attacked the ester of the laevo-form of mandelic acid more rapidly. To explain this apparent contradiction they postulated that there are two processes with distinct reaction rates, first the formation of the ester-ferment compounds and secondly the actual hydrolysis of the esters themselves.

However this may be, there are probably in the body optically active substances capable of forming compounds with mandelic acid without destruction of the stereochemical identity of the acid. If the formation and subsequent destruction of such compounds have different reaction rates according to the nature of the mandelic acid concerned, then in some such way may be explained the rapid initial preferential excretion of the laevo-form of mandelic acid.

Summary

1. *r*-Mandelic acid was given intravenously to decerebrate cats, either as the sodium salt or as the free acid.

2. The urine was collected fractionally and the mandelic acid recovered with little contamination by extraction of the urine with ether.

3. The early samples of urine had a marked excess of the laevo-form of the acid. In subsequent samples of urine the preponderance of the laevo-form of the acid became progressively less. In still later samples the recovered material was optically inactive, and ultimately, in experiments lasting a sufficient length of time, the samples of excreted material showed a steadily increasing dextro-rotation. A partial resolution was thus effected without the agency of bacteria.

4. The total excreted material in long experiments showed a rotation only slightly to the laevo-side.

5. The total amount of recovered acid in long experiments was between 80 and 90 % of the acid administered.

6. When pure active dextro- or laevo-acid was administered, the rotation of the recovered material was slightly below the theoretical value.

7. Possible interpretations of these results are discussed.

We are indebted to the Carnegie Trust for the Universities of Scotland for the purchase of apparatus used in the physiological side of the work, and to Imperial Chemical Industries for a grant to the Chemistry Department.

REFERENCES

Clough (1918). *J. chem. Soc.* **113**, 526.
Dakin (1903, 1). *Proc. chem. Soc.* p. 161.
—— (1903, 2). *J. Physiol.* **30**, 253.
—— (1905). *J. Physiol.* **32**, 199.
—— (1922). Oxidations and Reductions in the Animal Body. London: Longmans, Green and Co.
Droller (1938). *Brit. med. J.* **2**, 657.
Garry & Smith (1938). *J. Soc. Chem. Ind.* **57**, 602.
Henderson & Rule (1939). *J. chem. Soc.* p. 1568.
Knoop (1905). *Beitr. chem. Physiol. Path.* **6**, 150.
McKenzie (1902). *J. chem. Soc.* **81**, 1402.
—— & Harden (1903). *J. chem. Soc.* **83**, 424.
Marckwald & McKenzie (1899). *Ber. dtsch. chem. Ges.* **32**, 2130.
Montenbruck (1938). Inaug. Diss. Univ. Kiel.
Neubauer (1909). *Dtsch. Arch. klin. Med.* **95**, 211.
—— & Fischer (1910). *Hoppe-Seyl. Z.* **67**, 230.
—— & Gross (1910). *Hoppe-Seyl. Z.* **67**, 219.
—— & Warburg (1910). *Hoppe-Seyl. Z.* **70**, 1.
Quick (1928). *J. biol. Chem.* **80**, 515.
Roger (1932). *J. chem. Soc.* p. 2168.
—— (1935). *J. chem. Soc.* p. 1544.
Scholz (1938). Inaug. Diss. Univ. Kiel.
Westenbrink (1936). *Arch. néerl. Physiol.* **21**, 433.
—— & Gratama (1937). *Arch. néerl. Physiol.* **22**, 326.
Willstätter, Kuhn & Bamann (1928). *Ber. dtsch. chem. Ges.* **61**, 886.
Wrede (1938). *Pharm. Ind.* p. 401. 15/7.

61. THE FERMENTATION PROCESS IN TEA MANUFACTURE

5. CYTOCHROME OXIDASE AND ITS PROBABLE ROLE

6. THE EFFECT OF DILUTION ON THE RATE AND EXTENT OF OXIDATIONS IN FERMENTING TEA LEAF SUSPENSIONS

By E. A. HOUGHTON ROBERTS

From the Tocklai Experimental Station, Indian Tea Association, Cinnamara, Assam, India

(*Received 30 January 1940*)

5. CYTOCHROME OXIDASE AND ITS PROBABLE ROLE

In Part III of this series [Roberts, 1939, 2] evidence was presented in favour of the view that the primary stage in the oxidative processes in tea fermentation was an enzymic oxidation of ascorbic acid. There was considerable experimental support for this view and the conception was in accord with Huszák's [1937] findings on the role of ascorbic acid oxidase in peroxidase plants. A mechanism for the fermentation process in tea was put forward, based on the assumption that ascorbic acid was the oxygen carrier in both normal respiration of the tea leaf and in the fermentation process. This mechanism proved quite satisfactory in explaining the broad outlines of the process but subsequent work has revealed several inconsistencies which make the original mechanism untenable. These inconsistencies are considered below.

Objections to the ascorbic acid oxidase theory of fermentation

A consideration of the original fermentation mechanism shows that the R.Q. cannot possibly be less than 0·5 unless a large proportion of the dehydroascorbic acid is removed from the system before it can function as a hydrogen acceptor in carbohydrate oxidation. This latter possibility however would be incompatible with the continuation of the tea fermentation. As the R.Q. of the fermentation process in aqueous suspension is found experimentally to be 0·35–0·40 some adjustment of the previously published theory is clearly called for.

The variation of the fermentation rate, as measured manometrically, with the amount of tea leaf tissue suspended in the Warburg vessels cannot be reconciled with the ascorbic acid oxidase theory of fermentation. This phenomenon has already been referred to briefly [Roberts, 1939, 2] and will be considered in greater detail in the following communication. The effect is due probably to mass action effects, involving coenzymes and oxygen carriers, which result in variations in the concentration of the reduced form of the oxygen carrier undergoing the primary oxidation. This conclusion enables us to explain the variation in the rate of uptake with dilution of the tissue, if the concentrations of oxygen carrier in the reduced form are insufficient to saturate its specific oxidase. It

has been shown previously that addition of excess ascorbic acid has no effect on the initial rate of O_2 uptake by fermenting tea leaf tissue, so that if ascorbic acid is the oxygen carrier in question, it must be present in high enough concentration to saturate its oxidase. Obviously one, at least, of these two hypotheses must be rejected.

In addition to these theoretical objections, there is now direct experimental evidence available which has shown that the oxidation of ascorbic acid does not precede that of the tannins during fermentation.

Tea leaf at the end of fermentation retains the greater part of its enzymic activity. This is deduced from the finding that addition of fresh substrate, in the form of a green leaf infusion, or as tea-tannin, results in a renewed O_2 uptake at very nearly the original rate [Roberts, 1939, 2; Harrison & Roberts, 1939]. Despite this, ascorbic acid is oxidized very slowly if added to fermented tea leaf, the rate of O_2 uptake being much lower than that recorded at the beginning of fermentation as is shown in Table 1.

Table 1

	10	30	40	60	120 min.
Control	97	224	253	273	309
2 mg. ascorbic acid	95	227	263	306	375
10 mg. „ „	97	232	260	284	335

Uptakes are recorded in μl. Each Warburg vessel contained 100 mg. finely minced tea leaf. The ascorbic acid was added 30 min. after the commencement of shaking, at which time the greater part of the tannin had been oxidized. All results are means of triplicates, T = 36°.

The failure to attain the original rate of O_2 uptake is not due to inhibition of ascorbic acid oxidation by tannin fermentation products as this possibility has been excluded by separate controls. Addition of an infusion of fully fermented leaf has no inhibiting effect upon the rate of oxidation of ascorbic acid by freshly minced tea leaf.

Experiments were also carried out to investigate the rate of O_2 uptake when ascorbic acid was added at various stages of fermentation. The results of one such experiment are shown in Fig. 1.

Addition of ascorbic acid produces no significant acceleration in the rate of O_2 uptake, but on the other hand this rate of uptake ceases to decrease with time, and is maintained for some time at the level prevailing at the time of addition of the ascorbic acid.

It is concluded from these experiments that ascorbic acid is oxidized by the primary oxidation product of tea tannin,

$$o\text{-Quinone} + \text{ascorbic acid} \longrightarrow \text{catechol} + \text{dehydroascorbic acid},$$

so that when this product is largely removed from the system, by condensation, ascorbic acid is no longer oxidized. These results are completely at variance with the view that ascorbic acid oxidation precedes tannin oxidation, and must be taken as strong evidence against the ascorbic acid oxidase theory.

In a private communication to the author, Lamb expressed doubts as to whether the ascorbic acid oxidase theory could apply to results obtained by him in Ceylon. Lamb [1937] had shown that enzyme preparations obtained by precipitating tea leaf juice with alcohol could bring about the oxidation of catechols and tea tannin directly. This was difficult to reconcile with the idea of ascorbic acid as the oxygen carrier in tea fermentation as Lamb's enzyme should be completely free from this substance. On the other hand, at the time when this

criticism was made, it had been found impossible to confirm any of the Ceylon findings under Assam conditions.

The simplest explanation of the Ceylon findings would be that catechol oxidase was the enzyme concerned in tannin oxidation, but against this we have our previous results [Roberts & Sarma, 1938; Roberts, 1939, 1] which indicate that the presence of this enzyme in tea leaf is highly unlikely.

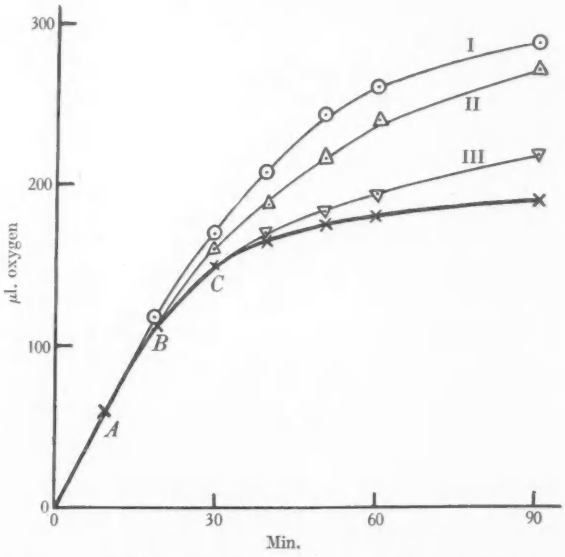


Fig. 1. Curves I, II and III show the rate of O_2 uptake subsequent to the addition of 2 mg. ascorbic acid after 10, 20 and 30 min. shaking respectively. The thick black curve shows the course of O_2 uptake by normally fermenting leaf without any addition of ascorbic acid. The uptakes plotted are the means of triplicates; each vessel contains 100 mg. minced tea leaf suspended in 3 ml. water.

A further difference of opinion arose when our first attempts at Tocklai to confirm Sreerangachar's results proved fruitless. Sreerangachar [1939] claimed that, after treatment of tea-leaf with acetone, and exhaustive washing of the residue with phosphate buffer, a considerable amount of "endo-enzyme" remained firmly bound to the tissue. This endo-enzyme could oxidize catechols or tea tannin directly. Despite a faithful adherence to Sreerangachar's methods it was found impossible to confirm these results at Tocklai, so long as the working up of the enzyme was carried out at our normal room temperatures. Peroxidase activity only could be detected in the final preparation. In the following section it will be shown how differences in laboratory temperatures were probably accountable for these contradictory findings.

The reconciliation of results from Ceylon and Assam provided fresh evidence against the ascorbic acid theory.

Effect of temperature during preparation on the activity of tea oxidase systems

A part of the evidence on which the ascorbic acid oxidase theory was founded was the relative instability of the enzyme responsible for the first stage of the fermentation process. Washing with alcohol or acetone, at 30°, was sufficient to destroy this enzyme completely.

The Tea Research Institute of Ceylon is in the hills and laboratory temperatures are considerably lower than they are at Tocklai. A possible explanation of the differences in opinion held by the biochemists of the two Institutes might be found in this difference in temperature. To test this, enzyme preparations from tea leaf were undertaken at normal room temperature (30–35°), and with refrigeration.

A fine mince of tea leaf was divided into two portions. One was ground with alcohol, at room temperature, and subsequently washed with successive small quantities of alcohol until the tissue was free from chlorophyll. The other portion was treated with alcohol in the same way, but during the whole process the system was cooled in ice-salt mixtures. After completion of washing, the tissue residues were dried *in vacuo* over H_2SO_4. The residue amounted to about 10 % of the original fresh weight of the tissue.

20 mg. portions of these tissue residues were suspended in 3 ml. of a green tea leaf infusion (20 g. leaf/250 ml. water) and O_2 uptakes recorded over a period of 3 hr. The results obtained are shown in Table 2; the uptakes recorded are the means of quadruplicates.

Table 2

	15	25	40	60	120	180 min.
Tissue residue washed at room temperature	23	28	32	40	55	67 μl. O_2
Tissue residue washed at 5°	62	96	136	184	282	324

The tissue residue, after washing with alcohol at 5°, retained about 35 % of the original enzymic activity of the green leaf. Washing with alcohol at room temperature, however, led to a much greater percentage inactivation of direct oxidase activity.

In the particular case just quoted, treatment with alcohol, at Tocklai room temperature, yielded a final product with about 7 % of its original oxidase activity. This is a lower degree of inactivation than is usually observed, and in some cases complete inactivation follows the alcohol treatment.

The above results provide conclusive proof that an oxidase system occurs in tea leaf which is readily inactivated by alcohol at temperatures of about 30°. It also explains our failure at Tocklai to confirm the findings of the Ceylon workers, as such inactivations will take place far more readily under our laboratory conditions, than they would at the Ceylon Tea Research Institute.

On the other hand, catechol oxidase is not likely to be inactivated under these relatively mild conditions, and it becomes necessary to consider what other oxidases may be present in tea leaf which can initiate the fermentation process.

The evidence for the presence of cytochrome oxidase in tea leaf

Four respiratory oxidases active in plant tissues have been described. Ascorbic acid and catechol oxidases have already been discussed and the possibility of their participation in tea fermentation has been rejected. There

remain dihydroxymaleic acid oxidase[1] [Banga & Szent-Györgyi, 1938], and cytochrome oxidase to be considered. The importance of the former as an aerobic oxidase cannot be assessed, and it would seem more likely that its function would lie in the metabolism of the C_4-dicarboxylic acids. The cytochrome system has recently been shown by Hill & Bhagvat [1939] to be present in green plants and its possible role in tea fermentation must be considered. As indophenol oxidase, this enzyme had been previously reported in some plant tissues [v. Euler, 1934].

It may be stated at the outset, that the assumption that the cytochrome system is concerned in tea fermentation enables us to account for all the experimental results obtained, both at Tocklai, and in Ceylon. A preliminary note to this effect has already appeared [Lamb & Roberts, 1939].

Cytochrome oxidase has long been known to be destroyed by alcohol and acetone. Battelli & Stern [1912] first demonstrated the destruction by alcohol and acetone of this enzyme, then known as phenylenediamine-oxydone and later as indophenol oxidase. The difficulty of getting the enzyme into solution has also long been recognized; it is very strongly bound to the cell structure. It is somewhat easier to obtain this enzyme in solution from plant tissues than from animal tissues but the affinity for the tissue is still high. Battelli & Stern also found that enzymic activity in O_2 was about three times that in air. This increase in rate of O_2 uptake with O_2 tension is not observed when the tissue is in a very fine state of division according to Hamburger & Szent-Györgyi [1925].

These characteristic properties of cytochrome oxidase are all shown by the tea leaf oxidase. The inactivation of the enzyme by alcohol has already been discussed, and Sreerangachar's results show how firmly the enzyme is attached to the tissue. Catechol oxidase on the other hand goes fairly readily into solution.

The rate of O_2 uptake by fermenting tea leaf is markedly accelerated by an increase in the oxygen tension. Table 3 shows the O_2 uptakes in μl. by 100 mg. of fermenting tea leaf in air and oxygen, as recorded in one experiment.

Table 3

Atmosphere	5	15	25	40	60 min.
O_2	79	161	184	198	207
Air	33	93	143	200	222

This experiment has been repeated many times and on each occasion the initial rate of O_2 uptake was two to three times as great in O_2 as it was in air. This was for finely minced leaf; with expressed tea leaf juice O_2 causes no significant acceleration in the rate of uptake as shown in Table 4.

Table 4

Atmosphere	5	15	25	40	60 min.
O_2	40	110	173	239	293 μl. O_2
Air	42	102	163	234	314

Each vessel contained 0·5 ml. tea leaf juice diluted to 3 ml. with water. Recorded uptakes are means of triplicates. Air was displaced by O_2 according to the usual method.

The colloidal matter in the juice expressed from tea leaf is in a very fine state of division. The failure of the rate of O_2 uptake to respond to substitution of the air by O_2 is reminiscent of Hamburger & Szent-Györgyi's findings for cytochrome oxidase activity in very finely divided tissue. Fleury [1924] reported

[1] It has recently been suggested that dihydroxymaleic acid oxidase is identical with peroxidase [Swedin & Theorell (1940)].

that in the case of laccase (now considered to be closely related to catechol oxidase) O_2 in any concentration exceeding 21 % had no influence on the rate of oxidation of substrates.

The enzyme preparations obtained from tea leaf by Lamb and the author would contain any cytochrome that was present in the leaf. Such enzyme preparations would therefore be expected to show very little specificity in regard to the substances whose oxidations they catalyse. Keilin & Hartree [1938] have shown that while cytochrome oxidase is markedly specific, the system oxidase + cytochrome-*c* + O_2 may oxidize catechol, quinol, *p*-phenylenediamine, ascorbic acid etc. The oxidized cytochrome is a powerful oxidizing agent, and acts upon any of the above substances without the assistance of an enzyme. Lamb's observations [1937] on the oxidation of catechol and tea tannin, which have recently been confirmed in this laboratory, and our own findings, that ascorbic acid and *p*-phenylenediamine are oxidized by fermenting tea leaf, may now be reconciled. It is now agreed that the probable cause of these oxidations is the cytochrome system [Lamb & Roberts, 1939].

The evidence which supports the theory that cytochrome oxidase is the enzyme responsible for the primary stage of fermentation is largely summarized below.

	Ascorbic acid oxidase	Catechol oxidase	Cytochrome oxidase	Tea oxidase
Treatment with alcohol or acetone	Inactivation	Nil	Inactivation	Inactivation
Affinity for tissue	—	Slight	Great	Great
Substrate specificity	Ascorbic acid	Catechols	(+cytochrome-*c*) catechols, tea tannin, *p*-phenylenediamine, ascorbic acid	Catechols, tea tannin, *p*-phenylenediamine, ascorbic acid
Effect of increased O_2 tension on activity	—	Nil	Marked acceleration when enzyme still adsorbed on tissue. In cell-free solution, nil	Marked acceleration when enzyme still adsorbed on tissue. In cell-free solution, nil

While the agreement between the properties of the tea oxidase and cytochrome oxidase is complete, we cannot say that this close parallelism proves that cytochrome oxidase is present. The possibility must be considered, that some respiratory enzyme is concerned, whose properties are very similar to those of cytochrome oxidase, but whose existence has not yet been recognized. Unfortunately, present conditions make it impossible to procure the necessary chemicals or apparatus to prove this point. Meanwhile, it will be assumed that cytochrome oxidase is present, as the experimental evidence is very strongly in favour of this view.

Discussion

The proof that ascorbic acid reduces the primary oxidation product of tea tannin enables one to explain the recent observations of Mituda [1938], that green tea contains more vitamin C than does black tea. One would expect the whole of the ascorbic acid to be oxidized during the course of fermentation, leaving black tea free from this vitamin.

The finding that cytochrome oxidase activity in plants may be destroyed by grinding with alcohol has an important bearing on Onslow's [1921] classification

of plant tissues. It is suggested, that a re-examination of plant tissues by the methods used in the case of the tea leaf, might lead to a more rigorous classification in terms of the respiratory enzyme contained in the tissue. In cases where oxidase activity was found by Onslow's methods, it is probable that catechol oxidase is concerned in the respiration. Where, however, peroxidase activity was reported, and oxidase activity was weak or absent, it seems likely that we are dealing with tissues in which cytochrome oxidase is the respiratory enzyme.

Apart from the substitution of cytochrome-*c* for ascorbic acid as the oxygen-carrier, it is not necessary to revise our ideas of the mechanism of tea fermentation very markedly. The following reaction scheme seems likely:

(1) Fe^{II}-haematin + O_2 $\xrightarrow{\text{cytochrome oxidase}}$ Fe^{III}-haematin + H_2O_2.[1]

(2) Tea tannin + Fe^{III}-haematin ----→ *o*-quinone + Fe^{II}-haematin.

(3) Tea tannin + H_2O_2 $\xrightarrow{\text{peroxidase}}$ *o*-quinone.

(4) *o*-Quinone -----→ condensation products.

(5) Fe^{III}-haematin + CoH_2 -----→ Fe^{II}-haematin + Co.

(6) *o*-Quinone + CoH_2 -----→ tea tannin + Co.

(7) Co + substrates $\xrightarrow{\text{dehydrogenases}}$ CoH_2 + reaction products.

The reduced and oxidized forms of cytochrome are written Fe^{II}- and Fe^{III}-haematin. Reduced and oxidized cozymase are represented by the symbols CoH_2 and Co.

Any dispersion of coenzymes would slow down reactions (5) and (6) by mass action effects.[2] Further, dispersion of the phosphate-carrying coenzymes would probably affect reaction (7) by decreasing the rate of formation of the triose-phosphates undergoing dehydrogenation. The combined effect of such coenzyme dispersions would be to increase the average mean life of the *o*-quinone molecule. If the *o*-quinone of tea tannin is not immediately reduced, it will undergo condensations, so that the assumption of coenzyme dispersion, following damage to the tissue, affords an explanation of the changes in tea tannin following damage to the leaf. Further evidence in favour of the coenzyme dispersion theory is presented in the following communication.

Summary

The oxidation of ascorbic acid by fermenting tea leaf is brought about by the primary oxidation product of tea tannin. Ascorbic acid oxidase is therefore not responsible for the first stage in the fermentation process.

The properties of the oxidase in tea leaf are compared with those of ascorbic acid, catechol and cytochrome oxidases, and complete agreement with those of cytochrome oxidase is found.

It is suggested that Onslow's classification of plants be revised, and that peroxidase plants should prove likely to contain the cytochrome oxidase system.

A revised scheme for the fermentation process is proposed based on the above findings.

[1] *Editor's note.* The direct formation of H_2O_2 in this reaction has never been demonstrated. There are, however, many other reactions from which H_2O_2 may arise in plants.

[2] Diaphorase may be active in reaction (5) but the introduction of this factor would needlessly complicate the argument.

6. THE EFFECT OF DILUTION ON THE RATE AND EXTENT OF OXIDATIONS IN FERMENTING TEA LEAF SUSPENSIONS

It has been suggested by the author [Roberts, 1939, 2], that the underlying cause of the irreversible oxidation and condensation of phenolic substances in plant tissues, which have suffered some form of damage, is the dispersion of coenzymes throughout the tissue. In the absence of supplies of coenzymes, which would enable this hypothesis to be tested directly, other methods of confirmation have been sought.

If one effect of rolling or mincing tea leaf is a dispersion of coenzymes, resulting in an effective dilution of these substances, suspension of finely minced tissue in water should still further dilute the coenzymes, and cause a further decrease in the rate of carbohydrate oxidation. Results have already been published [Roberts, 1939, 2] which support this conclusion. Carbohydrate oxidation was shown to be more extensive at lower dilutions of tissue, as the development of CO_2 was significantly greater for a suspension of 200 mg. tea leaf tissue in 3 ml. of water than for a suspension of 100 mg. in the same volume. In addition to a relatively lower rate of carbohydrate oxidation at high dilutions, the rate of O_2 uptake per unit weight of tissue is observed to be greater than when larger amounts of tissue are employed. In view of the possibility of obtaining confirmatory evidence for the coenzyme dispersion hypothesis of tea fermentation, and of the intrinsic interest of these observations, experiments have been carried out on a much wider scale.

To obtain results of any accuracy for CO_2 development during fermentation, it is necessary to take the mean of at least four determinations of O_2 uptake with and without KOH. This requires the use of eight Warburg vessels for each CO_2 determination. Obviously this method is not well adapted for a series of such determinations when using different dilutions of tea leaf tissue, as at least 40 vessels would be required simultaneously. Replication in time, although possible, is not considered desirable.

If the period of shaking be prolonged to $2\frac{1}{2}$ hr., oxidation comes practically to a standstill. Since tea tannin takes up 1 atom of O_2 per molecule, it may be assumed that any excess uptake is due mainly to carbohydrate oxidation. Variations in the extent of carbohydrate oxidation will be associated with variations in the total O_2 uptake, as the amount of O_2 consumed in tannin oxidation will be a constant, independent of the dilution of the tissue, so long as shaking is prolonged until complete oxidation of the tannins is achieved. Fully fermented leaf always contains some glucose, so that carbohydrates, unlike the tannins, are not completely oxidized during fermentation. Variations in the total amount of carbohydrate oxidized during fermentation are therefore understandable. The justification of the above method lies in the finding that the two methods yield comparable figures for the CO_2 output at different dilutions.

Variation of oxygen uptakes with dilution of the tissue

In Table 1 are recorded O_2 uptakes for fermenting tea leaf tissue suspended in water. The rates are calculated per 100 mg. of tissue (wet weight). Each figure is the mean of duplicates.

The results of only one such experiment are quoted, but, as in the other experimental results discussed below, this is fully representative of several determinations. No result is included which has not been repeated at least three times, and many of the experiments have been carried out a dozen or more times in all.

Table 1

Weight of tissue mg.	Volume of suspending fluid ml.	5	15	25	40	60	90	120	150 min.
84	4	59	141	173	179	188	198	206	206 μl. O_2
129	3	45	129	173	187	196	207	222	223
199	3	39	113	183	209	223	235	247	251
314	2	29	83	139	197	227	246	258	263
408	2	21	62	105	155	209	246	260	267

As the concentration of the tissue increases, the initial rate of O_2 uptake decreases, but the total uptake, after $2\frac{1}{2}$ hr., increases.

Relation between $-Q_{O_2}$ and tissue concentration

So long as the tissue concentration is greater than about 4 mg. dry weight per ml. of suspending fluid, $-Q_{O_2}$ increases with dilution. Below this concentration, the enzyme surfaces are presumably not saturated by their substrates, and further dilution decreases the rate of O_2 uptake.

Fig. 1 shows a typical relation found between $-Q_{O_2}$ and the tissue concentration. A straight line relationship is obtained by plotting $\log -Q_{O_2}$ against the log of the tissue concentration. It will be observed (Fig. 2) that, at the highest dilutions, values for $\log -Q_{O_2}$ do not lie on this straight line. The straight line has the general formula

$$\log (-Q_{O_2}) = b - a \log (\text{concn. tissue}).$$

The mean value for "a" from twelve different experiments is 0·41 with a standard error of 0·01 only. These twelve different experiments included cases of good and bad fermenting varieties of tea leaf (associated with high and low cytochrome oxidase contents), fresh leaf and withered leaf, and leaf of varying degrees of coarseness of plucking. The constant "b" gives the cytochrome oxidase activity where the tissue concentration is 1 mg. dry weight per ml., assuming no diminution in enzymic activity at these high dilutions.

The constancy of "a" enables us to compare the cytochrome oxidase contents of two samples of leaf by manometric determinations of the rate of uptake so long as the concentrations of the two samples are the same. It is convenient to have some standard concentration for $-Q_{O_2}$ determinations, and the one adopted is of 15 mg. dry weight per ml. of water. This corresponds to about 200 mg. fresh green leaf suspended in 3 ml. water; the exact quantity will vary with the moisture content of the leaf. Values for $-Q_{O_2}$ in future will be given for this standard concentration unless otherwise stated.

The marked decrease in the rate of O_2 uptake with concentration enables us to explain the relatively slow rate of fermentation under factory conditions. Here the rolled leaf is spread at a thickness of about an inch on the fermenting room floor. Including the time spent in the rollers, the leaf requires about 3 hr. for fermentation, whereas in the Warburg vessel a similar extent of oxidation takes place in 1 hr. only. The rolled leaf contains appreciable amounts of water (say 65 %), in which case it may be considered as a suspension containing 35 g. of solid matter in 65 ml. of water. The value of $-Q_{O_2}$ for this concentration may

be obtained by extrapolation, and it is found to be just about one-third of that determined under the standard conditions. It is possible therefore, to account

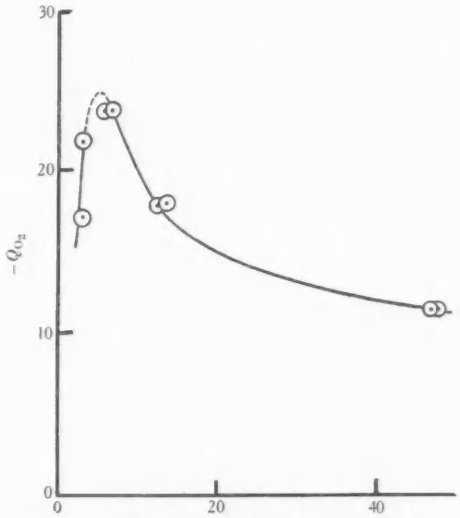


Fig. 1.

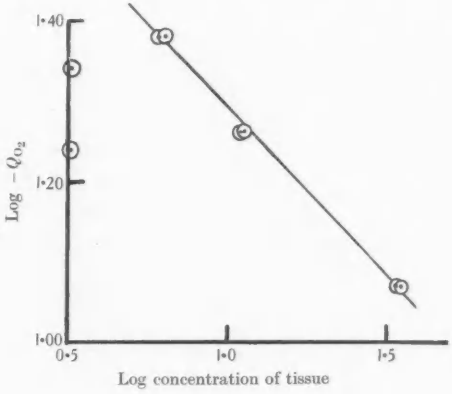


Fig. 2.

for the much slower rate of fermentation under factory conditions, entirely in terms of the phenomenon under consideration, although other factors are almost certain to make their presence felt.

Relation between total uptake and tissue concentration

As has already been shown in Table 1, the total O_2 uptake per unit amount of tissue, when oxidation has come nearly to a standstill, increases with the con-

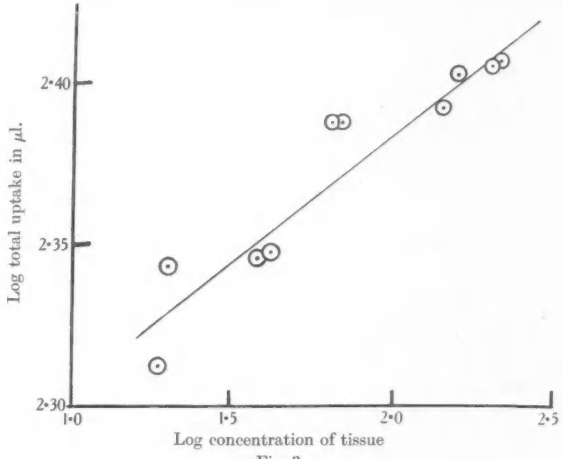


Fig. 3.

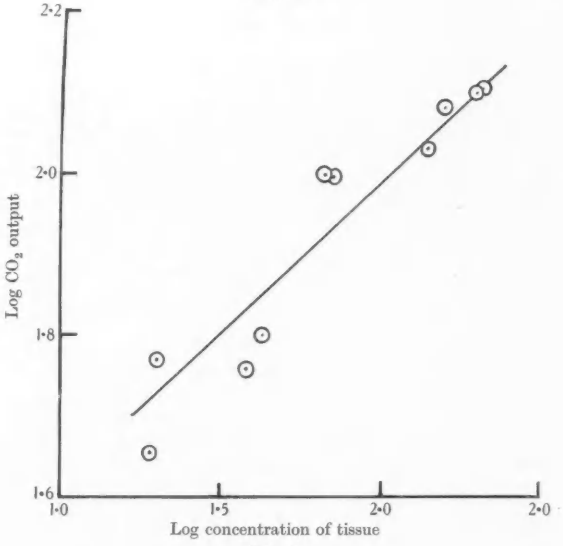


Fig. 4.

centration of the tissue suspension. By plotting logs of the total uptake, against logs of the tissue concentration, we obtain quite good approximation to a straight

line (Fig. 3). Knowing the amount of O_2 utilized in tannin oxidation, the CO_2 outputs can be calculated and a similar straight line relationship is found between log (CO_2 output) and the log of the tissue concentration (Fig. 4).

The greater degree of oxidation of carbohydrates at higher tissue concentrations is evident if we accept the view that CO_2 originates largely in carbohydrate breakdown.

By extrapolating both the above curves to points corresponding to green leaf, approximate values may be obtained for total O_2 consumption and total CO_2 evolution for leaf undergoing fermentation under factory conditions. The total O_2 requirement under normal factory conditions is thus found to be about 20–25% higher than that found in the Warburg vessel under standard conditions. Further, the R.Q. is found by calculation to be 0·50–0·55 for normal fermentation, against the values of 0·35–0·40 that were determined manometrically.

Interpretation of results

Diffusion factors

It may be stated at the outset that the variations in the rate of uptake are not due to various diffusion effects such as those studied by Dixon & Elliott [1930], as uptakes are not affected by the rate of shaking. This interpretation of the above results is also excluded by experiments with tea leaf juice.

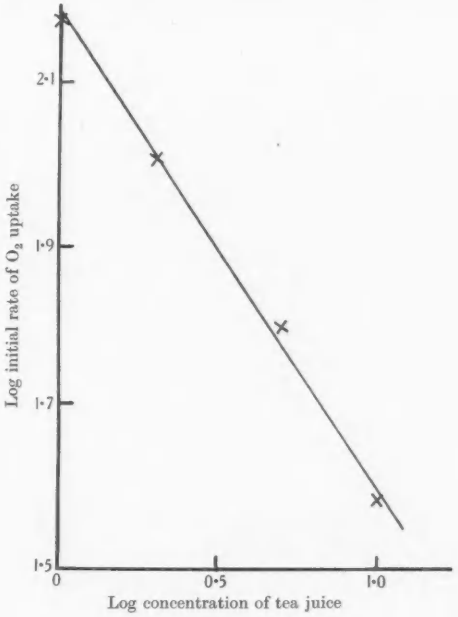


Fig. 5.

Juice may readily be expressed from coarsely minced tea leaf and this juice undergoes a similar fermentation to that of bruised leaf. Oxygen is consumed,

some CO_2 is produced, and tannins are oxidized. The rate of O_2 uptake is affected very much by the dilution as is shown in Table 2.

Table 2

Volume of juice ml.	5	15	25	40	60	120 min.
0·1	15	25	34	42	60	98 μl. O_2
0·2	20	36	52	73	100	165
0·5	32	72	104	150	206	328
1·0	39	70	151	224	313	510

Total volume = 3 ml., T = 36°. All figures are means of duplicates in good agreement.

The initial rate of O_2 uptake with 1 ml. of juice is only two or three times as great as that recorded with 0·1 ml. juice, despite a tenfold increase in enzymic concentration. As with tissue minces, the rate of reaction per unit quantity of enzyme decreases as the concentration of the enzyme increases. Plotting logs of the initial rate per ml. juice against logs of the concentration of the juice we obtain a straight line (Fig. 5) similar to that obtained in the case of fine minces of tea leaf.

The same effect therefore is obtained with both a fine mince and a cell-free extract such as tea leaf juice. Diffusion effects, where the permeability of the the tissue is a limiting factor, therefore, can be excluded.

Substrate concentration

According to the classical views of Michaelis and others, substrate concentration has two important effects on the rate of an enzymic reaction. In sub-optimal concentration the reaction rate may be lowered, owing to incomplete saturation of the enzyme surface by the substrate. On the other hand, considerable excess of substrate may cause inhibition of activity, and this is especially likely with a substrate such as tea tannin.

In the normal range of concentrations used in these experiments neither of these factors operates. It has already been shown that the initial rate of reaction is unaffected by the addition of a green leaf infusion, or of a fresh tea tannin preparation [Roberts, 1939, 2], and later work has confirmed this observation at several concentrations. As fresh tea tannin causes neither acceleration nor retardation of the process, neither of the above substrate concentration effects can be responsible for the variation in the rate with tissue concentration.

Simple oxidase-substrate systems

If we assume the dilution effects, under consideration in this communication, to be due to variations in the degree of dispersion of the coenzymes required for carbohydrate breakdown, no such effects should be noticeable when dealing with a simple oxidation of tea tannin by the cytochrome system.

An enzyme preparation was obtained by precipitating the expressed juice of withered tea leaf with alcohol at *ca.* 5°. Suspensions of this preparation in phosphate buffer were incubated with aqueous solutions of tea tannin [Harrison & Roberts, 1939] in Warburg vessels, and the rate of oxidation followed manometrically. Concentration was found still to have some effect on the rate of reaction, but this was found to be due to the inactivating effect of the substrate in high concentrations. The enzyme, after partial purification, is much more susceptible to tea tannin. A similar observation with respect to peroxidase has previously been made [Roberts & Sarma, 1938].

However, if pyrogallol be used as the substrate, a range of concentrations may be found where rate of reaction per unit amount of enzyme is independent of enzyme concentration. It has been shown previously, by Harrison & Roberts [1939], that pyrogallol may function as an oxygen carrier in carbohydrate oxidation.

100 mg. of the enzyme preparation were suspended in 10 ml. of *p*H 5·8 *M*/5 phosphate buffer. Mixtures of the above enzyme solution were made up with varying quantities of a pyrogallol solution, containing 510 mg. in 100 ml. of water, according to the following table, adjusting the total volume of 3 ml. with water in each case.

Enzyme ml.	Substrate mg.
2	5·1
1	2·55
0·5	1·28
0·25	0·64

Uptakes were measured over a period of 2 hr. as recorded in Table 3.

Table 3

Enzyme ml.	5	15	25	40	60	120 min.
2·0	102	294	449	678	810	938 μl. O_2
1·0	82	224	320	402	430	464
0·5	44	114	166	210	221	232
0·25	21	54	76	98	107	111

All figures are means of duplicates in good agreement.

Initial rates in μl. O_2 per ml. enzyme per hr. $(-Q_{O_2})$.

Enzyme ml.	$-Q_{O_2}$
2·0	612
1·0	984
0·5	1056
0·25	1008

At the three lower concentrations of enzyme and substrate, variation in concentration has no significant effect on the rate of reaction. At the highest concentration however the substrate concentration seems to have some inhibiting effect. Otherwise, this experiment shows quite clearly that where variations in coenzyme concentration can have no effect on the rate of reaction, enzyme concentration can vary between certain limits without affecting the rate of reaction per unit quantity of enzyme.

The total uptakes show that 2·0 atoms of oxygen are consumed in the oxidation of each pyrogallol molecule. No purpurogallin is formed. On the other hand, it was shown by Harrison & Roberts that, when pyrogallol was added to fermenting tea leaf, only 1 atom of oxygen was used up in the oxidation of each molecule of pyrogallol. To account for this difference, it is suggested that the primary product of pyrogallol oxidation can enter into combination with other constituents of the fermenting tea leaf.

With catechol oxidase, pyrogallol oxidation is accompanied by an uptake of 3 atoms of oxygen and the separation of the product, purpurogallin, in crystalline form [Willstätter & Heiss, 1923]. This represents a further distinction between the tea oxidase and catechol oxidase.

Discussion

Coenzyme dispersion on rolling

The above evidence, on the whole, supports the coenzyme dispersion theory. Diffusion and substrate concentration factors have been excluded and, where coenzyme dilution can have no effect, the phenomenon under discussion is no longer observed. Variations in the extent of carbohydrate oxidation are predictable in terms of coenzyme dispersion at varying dilutions.

Direct proof of the role of coenzymes in tea fermentation is difficult under the present circumstances, and this indirect evidence in favour of the theory is consequently very welcome.

The literature on the effect of damage on tissue respiration is steadily growing but four main effects seem distinguishable.

(1) Damage to tissues may result in an intermingling of coenzymes and enzymes, such as phosphatases and nucleotidases, capable of bringing about the breakdown of coenzymes. In this case, coenzyme activity would become progressively smaller throughout the course of tea fermentation. As the ratio of carbohydrate oxidation to total oxidation remains constant throughout the whole process, this mechanism cannot be operative in tea fermentation.

(2) Geiger [1939] showed that cytolysis destroyed both glycolytic activity and respiratory power in brain tissue. This he showed to be due to the liberation of an inhibitor which was enzymic in nature. The factor destroyed by this inhibitor, however, was not one of the coenzymes. This phenomenon must be distinct from that observed in fermenting tea leaf, as with suspensions of fermenting brain pulp, carbohydrate breakdown is accelerated by dilution, and not retarded.

(3) According to Yudkin [1937], the lysis of *M. lysodeikticus* by egg-white lysozyme destroys dehydrogenase activity. The enzymic activity is probably dependent upon the intactness of the bacterial cell-wall, for its activity is not restored by addition of coenzymes.

(4) In the opinion of the author, damage to cellular material may result in some dispersion of coenzymes, and hence to a reduction in their effective concentration.

In the case of the tea leaf it is considered that the effect of rolling on the leaf is a disorganization of the contents of the cell. There is no actual visible rupture of the cell-wall, but the effect of rolling may well be on the complex protein-lipoid interphase between protoplasm and vacuole. Extension or compression of cells may bring about considerable changes in the orientation of molecules in this interphase, and hence of their apparent energy of activation. Not only may this reduce the chemical activity of the coenzymes, but the affinity of the coenzymes for the active surface may be reduced, so that an actual dispersion of coenzymes into the aqueous phase also may occur.

Variation of $-Q_{O_2}$ *with tissue concentration*

In the scheme of reactions put forward to explain the fermentation process, the only one in which oxygen is concerned is the enzymic oxidation of cytochrome. Variations in the rate of O_2 uptake per unit amount of tissue, therefore, are in all probability due to variations in the substrate concentration. This requires that the amount of cytochrome in the reduced form is insufficient to saturate its oxidase.

A comparison of the reactions taking place in fermentation under factory

conditions, and in suspensions of 10–15 mg. minced tea leaf tissue (dry wt.) per ml., enables some explanation of these results to be made.

It was shown by Roberts & Sarma [1938] that excess catalase inhibited fermentation. These observations were carried out on coarsely minced tea leaf mixed with just sufficient water to allow easy access of the catalase preparation to the fermenting leaf. It must be concluded from this observation that under such conditions oxidized cytochrome does not oxidize tea tannin to any extent, and that the bulk of the oxidation must take place by peroxidase and H_2O_2. We may therefore write down the essential reactions which follow cytochrome oxidation as follows:

(1) Tea tannin + H_2O_2 $\xrightarrow{\text{peroxidase}}$ *o*-quinone.

(2) Fe^{III}-haematin + CoH_2 ⟶ Fe^{II}-haematin + Co.

(3) *o*-Quinone + CoH_2 ⟶ tea tannin + Co.

In dilute suspensions of finely minced tea leaf, it has been shown that catalase has no significant effect on the rate of O_2 uptake ($-Q_{O_2}$ in both cases $20{\cdot}0 \pm 0{\cdot}3$). It may be concluded that, in such suspensions, the dilution of H_2O_2 is such that peroxidase activity is considerably diminished. Further, carbohydrate oxidation proceeds only so long as any tea tannin remains unoxidized [Roberts, 1939, 2]. At these dilutions the pyridine nucleotide coenzymes are possibly dispersed too much to be oxidized, to any great extent, by the oxidized cytochrome. The *o*-quinone of tea tannin will, in this case, act almost exclusively as the O_2 carrier required to oxidize the coenzymes. The essential reactions in dilute, minced tea leaf suspensions may therefore be written:

(1) Fe^{III}-haematin + tea tannin ⟶ Fe^{II}-haematin + *o*-quinone.

(2) *o*-Quinone + CoH_2 ⟶ tea tannin + Co.

In these two distinctly different reaction mechanisms the proportions of the cytochrome in the reduced form will almost certainly be different. This proportion unfortunately cannot be worked out without a knowledge of the equilibrium constants, Michaelis constants and other such data pertaining to the various reactions. No sudden change over from one mechanism to another on dilution is visualized, but it seems that as the dilution proceeds the latter mechanism will gradually preponderate. Each particular dilution is therefore likely to be associated with a characteristic value for $\frac{[Fe^{II}\text{-haematin}]}{[Fe^{III}\text{-haematin}]}$ and hence with a particular value for $-Q_{O_2}$. Whether this ratio, and $-Q_{O_2}$, should increase or decrease with dilution cannot be predicted from the data at our disposal. Further work on this aspect of the subject is therefore desirable, but cannot be undertaken at Tocklai for the present.

Summary

With suspensions of fermenting tea leaf in water, it is found that, at higher dilutions, the rate of O_2 uptake increases, while the rate of carbohydrate oxidation decreases. These observations are considered in terms of the coenzyme dispersion theory of fermentation.

The various effects of damage on cellular respiration are considered, and that of coenzyme-dispersion shown to be the one which best explains the phenomena of tea fermentation.

Tea fermentation, in dilute aqueous suspension, differs in some important respects from the process as carried out in the factory. In dilute suspensions peroxidase plays a negligible role.

The author wishes to thank the Director, Mr P. H. Carpenter, and his colleagues for their continued interest in this work and for their useful criticisms. Thanks are also due to the Indian Tea Association for permission to publish these results, and to Messrs P. B. Sen Gupta, S. N. Sarma and D. N. Barua for the continued high standard of their technical assistance.

REFERENCES

Banga & Szent-Györgyi (1938). *Hoppe-Seyl. Z.* **255**, 57.
Battelli & Stern (1912). *Biochem. Z.* **46**, 343.
Dixon & Elliott (1930). *Biochem. J.* **24**, 820.
v. Euler (1934). *Chemie der Enzyme*, **2**, pt. III, 403.
Fleury (1924). *C.R. Acad. Sci., Paris*, **178**, 1027.
Geiger (1939). *Biochem. J.* **33**, 377.
Hamburger & Szent-Györgyi (1925). *Biochem. J.* **157**, 298.
Harrison & Roberts (1939). *Biochem. J.* **33**, 1408.
Hill & Bhagvat (1939). *Nature, Lond.*, **143**, 726.
Huszák (1937). *Hoppe-Seyl. Z.* **247**, 239.
Keilin & Hartree (1938). *Proc. roy. Soc.* B, **125**, 171.
Lamb (1937). *Tea Res. Inst. Ceylon, Ann. Rep.* 1937, 65.
—— & Roberts (1939). *Nature, Lond.*, **144**, 867.
Mituda (1938). *J. agric. chem. Soc. Japan*, **14**, 1228.
Onslow (1921). *Biochem. J.* **15**, 107, 113.
Roberts (1939, 1). *Biochem. J.* **33**, 836.
—— (1939, 2). *Biochem. J.* **33**, 842.
—— & Sarma (1938). *Biochem. J.* **32**, 1819.
Sreerangachar (1939). *Curr. Sci.* **8**, 13.
Swedin & Theorell (1940). *Nature, Lond.*, **145**, 71.
Willstätter & Heiss (1923). *Liebigs Ann.* **433**, 17.
Yudkin (1937). *Biochem. J.* **31**, 1065.

LINDA HALL LIBRARY
KANSAS CITY, MO

62. ENZYME SYSTEMS IN ARTICULAR CARTILAGE

By CECILIA LUTWAK-MANN[1]

From the Biochemical Laboratory, Cambridge

(*Received 25 January 1940*)

Early research on cartilage was chiefly concerned with the chemical composition of this tissue (mineral content, collagen, chondroitin sulphuric acid). The study of enzymes in cartilage was inaugurated by Robison [1923] who found that cartilage extracts liberate inorganic phosphate from hexosephosphoric esters. Much valuable work has been done since with regard to the phosphatase content of cartilage especially in relation to rickets, but comparatively few attempts have been made to study other enzyme systems in cartilage. Recently Bywaters [1937] carried out a series of experiments on joint tissues of the horse and determined manometrically the respiration and glycolysis quotients of horse cartilage.

In the present study the dephosphorylating enzymes were reinvestigated mainly with the object of learning more about the intermediary and end-products of various substances dephosphorylated by cartilage. Attempts were also made to find out whether there are in articular cartilage any oxido-reduction systems analogous to those known to play an essential part in the respiration and glycolysis of other tissues.

Experimental

Material. Articular cartilage was used throughout this work. Calf carpal joints were obtained from the slaughterhouse as soon as possible after the death of the animal. Only material from normal young animals was used and inflammatory changes or other lesions were met with in not more than a dozen out of over 200 joints examined.

The joints were opened in the laboratory, the synovial fluid drained off and cartilage removed from the joint surface with a sharp knife in the form of thin slices ("shavings") 0·4–0·8 mm. thick. After thorough rinsing with cold sterile water to remove traces of synovial fluid and blood, the slices were used for experiments either (1) directly, a weighed amount being suspended in 3–5 volumes of fluid, or (2) extracts were prepared by incubating cartilage at 37° for 2 days with 5 times its weight of water and a few drops of toluene or octyl alcohol. Liquid air treatment was at first applied to reduce cartilage to a powder. Later, however, it was abandoned as it offered little advantage over the use of finely chopped tissue.

When cartilage slices were used directly without previous maceration in presence of an antiseptic, little time was allowed to elapse between the removal of the tissue from the joint and the onset of the experiment, so as to avoid bacterial contamination. Experiments without a disinfectant were run for not longer than 8 hr. at 37°. Bacterial examinations were made whenever there was any suspicion of the possibility of excessive bacterial growth which might influence the results.

Various fluids were tested for the preparation of cartilage extracts, such as saline, 0·9 % KCl, dilute phosphate, glycerol. Simple extraction with water was,

[1] Research Fellow, Newnham College, Cambridge.

however, found to give the most satisfactory and constant results especially because the total volume of the water added could be recovered after incubation, which was not the case with, e.g., dilute phosphate which caused variable and considerable swelling of the cartilage. The aqueous extract is colourless, slightly opalescent and has a *p*H of 7·6. It preserves its properties for a long time at 0°. A number of such extracts was tested and relatively little variation was found in their content of dry matter (0·2 %), total N (5·5 mg./100 ml.), inorganic P (8–10 mg./100 ml.), Mg (0·15 mg./100 ml.) and NH_3-N (0·1–1 mg./100 ml.). On the other hand, somewhat more variation was found in their enzymic activity. A second extraction of the cartilage residue was of little value. In all experiments described below the extracts were prepared from fresh tissue but it was possible to use successfully vacuum-dried cartilage for preparing active phosphatase extracts even after keeping several weeks in the dry state.

On several occasions experiments were carried out with synovial fluid and a few were done with synovial mucosa and ligaments. Synovial mucosa was used directly in the form of small scraps, whereas ligaments were cut into little pieces and treated with liquid air, which caused their disintegration into single fibres. These swell enormously and retain most of the added fluid so that it was rather difficult to obtain an extract from ligaments.

Occasionally experiments were carried out with cartilage from bullock joints. The amount of material which can be obtained from the joint surface of a grown animal is about one tenth of that from a young one. In spite of the striking anatomical differences, cartilage from normal adult animals appeared to possess the same enzyme systems as that of growing animals.

Methods. Phosphate estimations were done by the Fiske and Subbarow method [1925] as modified by Lohmann & Jendrassik [1926]. In experiments with cartilage extracts the method was applied in the ordinary way after removal of proteins with trichloroacetic acid. When cartilage slices were used, the aqueous solution in which they were suspended was poured off, the residue washed twice with a few ml. of water, and the combined washings were precipitated with trichloroacetic acid. This procedure gave better results than if the acid was added to the sample containing cartilage. It was found that a single extraction of sliced cartilage with trichloroacetic acid yielded only about half of the total acid-soluble P-content and further the amount of P varied greatly with the duration of the acid extraction. To exhaust the P-content of cartilage it was necessary to repeat the trichloroacetic acid treatment at least three times, each extraction lasting several hours. On the other hand, it was established in a large number of experiments that the amount of P which diffuses from minced cartilage into an aqueous solution is comparatively small and varies little from one experiment to another. It is realized that P-values obtained by the above procedure are diminished by the retention of a certain amount of P in the cartilage slices. However, the results can be considered satisfactory for the purpose of comparing the extent of dephosphorylation of various esters in a given time at 37°, provided that the amount of tissue and fluid and the molar concentration of the substrates is kept unaltered. The routine composition of the experimental samples was 1 g. tissue, *M*/20 substrate, total volume 5 ml.

Ammonia determinations were carried out by distillation with borate in the Parnas & Heller apparatus [1924] with subsequent nesslerization.

Lactic acid was estimated by the method of Friedemann *et al.* [1927] and occasionally by the colorimetric method of Mendel & Goldscheider [1925]. Proteins were precipitated with tungstic acid and interfering substances removed by means of the copper-lime treatment. Care was taken not to have in any

sample more than 3–4 mg. of substances such as acetate, iodoacetate, pyruvate or salicylate, because it was found that in excess they decrease the yield of lactic acid estimations by 30–40 %.

Bisulphite-binding substances (B.B.S.) were determined by the method of Clift & Cook [1932], glycogen by Pflüger's method, glucose by ferricyanide reduction [Hagedorn & Jensen, 1923], carbohydrates by the orcinol method of Tillmans & Philippi [1929], ascorbic acid by titration with 2:6-dichlorophenol-indophenol [Birch *et al.*, 1933] and glutathione iodometrically.

For manometric measurements, which were carried out in Barcroft manometers with side bulbs and gas outlets, fresh and very finely sliced cartilage was used, not exceeding 0·8 g. wet weight of tissue (78 % water) per 3·5 ml. total volume. A number of experiments was carried out by means of the Thunberg technique with 1 g. tissue per tube and 1·0 ml. *M*/2000 methylene blue in a total volume of 4 ml.

The various phosphoric acid esters and coenzymes used in the experiments were prepared in the Laboratory according to current methods, except hexose-diphosphate, adenylic acid and α-phosphoglycerol, which were commercial preparations. They were all used in the form of their Na or K salts.

Results

In order to gain more insight into the glycolysis and respiration of normal articular cartilage it was thought necessary to analyse in detail the effect of this tissue upon various substances which are known to be closely connected with these processes in other tissues. Experiments will be described below on the behaviour of various phosphorylated and non-phosphorylated components of the glycolytic cycle under the influence of cartilage. A separate section will contain the results obtained from the study of other enzymic oxidations and reductions in cartilage.

1. *Decomposition of adenine derivatives.* Cartilage as well as cartilage extract possesses enzymes which dephosphorylate muscle adenylic acid very rapidly, yeast adenylic acid a little less vigorously and adenylpyrophosphoric acid more slowly still. The liberation of phosphate is accompanied by deamination, for instance 0·9 mg. P and 0·38 mg. NH_3-N are found after 3 hr. incubation of 10 mg. muscle adenylic acid with 1 g. cartilage at 37°. In similar conditions 60 % of yeast adenylic acid is decomposed and not more than 20 % of adenosinetriphosphoric acid. It appears that the enzyme responsible for the deamination is more soluble than the phosphatase: if minced cartilage is macerated for a few hours with 2–3 volumes of water and this extract is then discarded, the residue still dephosphorylates, but scarcely deaminates, muscle adenylic acid. Of other substances closely related to those mentioned above, inosinic acid is dephosphorylated and adenosine deaminated by cartilage. Ammonia is also liberated from adenine but only extremely slowly.

The end-product of decomposition of all these compounds by calf cartilage is hypoxanthine. This was shown both by the chemical isolation of the characteristic hypoxanthine silver-picrate derivative as well as by the use of a purified milk xanthine oxidase preparation.

The fact that it is possible to demonstrate the presence of hypoxanthine after several hours' incubation of adenine derivatives or hypoxanthine itself with cartilage at 37°, makes it rather improbable that normal (calf) cartilage is capable of metabolizing this purine to any great extent. Nevertheless, it should be recorded that on a few occasions it was possible to observe a slight but definite

oxidation of hypoxanthine by cartilage slices (not however, by extracts) both in aerobic and anaerobic conditions (see also p. 525).

The effect of cartilage upon more complex adenine derivatives such as cozymase and Warburg's coenzyme was also examined. The experiments were arranged in the following way. The coenzyme solutions were incubated with cartilage at 37° and after a certain time their presence was tested for by a suitable enzyme system such as the lactic dehydrogenase (for cozymase) and the hexose-monophosphate dehydrogenase (for Warburg's coenzyme). As controls, coenzyme solutions were put up with boiled tissue. It was found that while a short incubation with cartilage leaves these substances unaffected, they are no longer able to display their coenzyme activity if acted upon by cartilage for a longer time. It may be added here that boiled juice (Kochsaft) from cartilage neither activates nor interferes with dehydrogenase activity.

While adenine derivatives are obviously unstable in presence of cartilage, guanylic acid, guanosine and guanine remain unchanged. It was also found that no substance other than adenine derivatives is deaminated by cartilage although several amino-acids as well as glutamine, asparagine, creatine, guanidine and glucosamine were tested.

2. *Decomposition of phosphorylated sugar derivatives.* The following phosphoric acid esters were examined and all found to undergo dephosphorylation in presence of calf cartilage, but to a varying extent: fructose-1:6-diphosphate (Harden & Young ester), fructose-6-monophosphate (Neuberg ester), glucose-1-monophosphate (Cori ester), 3-phosphoglycerate and α-phosphoglycerol. At *p*H 7·6 Cori ester is dephosphorylated most rapidly, next follows Neuberg ester, then Harden & Young ester; in standard conditions at 37° and after 4 hr. the whole P of the first, 50 % of the second and 20 % of the last ester, being liberated. Phosphoglyceric acid yields inorganic P slowly unless suitable acceptors are provided (see below) and α-phosphoglycerol gives off only about 20 % of its P-content after several hours of incubation with calf cartilage.

Cori ester. A ferricyanide-reducing sugar is formed on dephosphorylation of this ester. A small proportion of the sugar formed is converted into lactic acid. The dephosphorylation of Cori ester by cartilage is inhibited by *M*/20 NaF but not completely. It may be mentioned here that generally it was found difficult to obtain NaF-effects in cartilage, even using comparatively high concentrations of the inhibitor and a long time of incubation. It is probable that this is due to the high content in this tissue of Ca and Mg.

Experiments were also arranged to test whether there is an uptake of inorganic phosphate by glucose and glycogen in presence of NaF in cartilage. However, only insignificant results were obtained even after prolonged incubations.

Neuberg ester. The dephosphorylation of this substance is usually accompanied by the production of small amounts of lactic acid. The reaction is not sensitive to NaF.

Harden & Young ester. Whereas the dephosphorylation of the two esters quoted above is independent of *p*H in the range 6·5–8·0, hexosediphosphate remains practically untouched unless the *p*H is above 7·4. Since the *p*H of cartilage is at least 7·6 and that of synovial fluid varies between 7·8 and 8·2, one can assume that suitable conditions exist normally for a rapid breakdown of this substance.

Adenosine, adenylic acid and pyruvate in small amounts were each tested as possible acceptors for the phosphate given off by fructosediphosphate but no significant results were obtained. Addition of Mg is without effect upon the

dephosphorylation of Harden & Young ester by whole cartilage which is itself rich in Mg. Cartilage extracts contain also a fair amount of Mg, practically all of which can be removed by dialysis. Even then, however, only slight acceleration is seen on addition of Mg. Similar observations with regard to the effect of Mg on the rate of dephosphorylation by cartilage were also made in the case of adenosine triphosphate and inorganic pyrophosphate (see below).

Iodoacetate, salicylate, bile salts and cinchophen (phenylcinchoninic acid) were also tested but appeared to have no marked effect upon the dephosphorylation of hexosediphosphate.

When the Harden & Young ester was acted on by aqueous toluene cartilage extracts, as the source of enzymes, it was observed that an orange hydrazone is formed in the trichloroacetic acid filtrate on addition of 2:4-dinitrophenylhydrazine. The hydrazone is insoluble in acid and carbonate and dissolves in alcoholic KOH with a deep blue-violet colour. Its M.P. is 298° which is that of the hydrazone of methylglyoxal. No such hydrazone is formed from hexosediphosphate after incubation with boiled cartilage. It was found later that formation of methylglyoxal from hexosediphosphate occurs also in fresh cartilage in absence of toluene so that it may be regarded as a normal intermediary of hexosediphosphate breakdown.

Quantitative estimations of the methylglyoxal formed were carried out by the method of Clift & Cook [1932]. Cartilage itself has no bisulphite-binding substances. It is impossible to determine exactly the relation between the amount of phosphate liberated from hexosediphosphate and the methylglyoxal formed, because the latter undergoes a further change in cartilage as will be reported below. When whole cartilage is used slightly more methylglyoxal is found in aerobic than in anaerobic conditions. Toluene treatment also increases the yield to some extent. Still more triose is found in presence of iodoacetate, whereas addition of GSH usually decreases the amount of bisulphite-binding substances (Table 1).

Table 1. *Decomposition of Harden & Young ester*

3·1 mg. Harden-Young ester (H.Y.)-P. 2 g. cartilage. 10 ml. veronal buffer *p*H 7·8, 4 ml. synovial fluid. Incub. 8 hr. 37°.

Additions		mg. P liberated	mg. B.B.S. (calc. as methylglyoxal)	mg. lactic acid	2:4-Dinitrophenylhydrazone
		Cartilage			
0		0·08	0	0·52	—
H.Y.	in air	2·10	0·90	3·52	+
,, + *M*/20 NaF	,,	0·15	0	0·12	—
,, + cozymase	,,	2·70	0·50	5·04	+
,, + toluene	,,	2·50	1·10	3·40	+ +
,, + GSH	,,	Not estimated	0·40	4·52	±
,, + *M*/50 iodoacetate	,,	2·00	1·45	3·52	+ +
,, anaerobic		2·30	0·60	3·90	+
		Synovial fluid			
0		0·02	0	2·30	—
H.Y. in air		0·15	0·66	4·80	+

Small amounts of lactic acid are found to be formed from hexosediphosphate in presence of cozymase. The reaction is considerably increased by the addition of pyruvate (Table 3).

Phosphoglyceric acid. As already stated inorganic phosphate is liberated from phosphoglyceric acid only very slowly. However, when small amounts of creatine and adenylic acid are added, the rate of dephosphorylation is markedly

accelerated. Creatine alone has a similar but much smaller effect. Pyruvic acid can be demonstrated as the product of dephosphorylation, both as a bisulphite-binding substance and by precipitation with 2:4-dinitrophenylhydrazine. NaF inhibits the reaction largely but not completely (Table 2).

Table 2. *Decomposition of phosphoglyceric acid*

1·5 mg. phosphoglyceric acid (pgl.)-P. 1 g. cartilage, 5 ml. veronal buffer *p*H 7·8. Incub. 12 hr. 37°.

Additions	mg. P liberated	mg. B.B.S. (calc. as pyruvate)	2:4-Dinitrophenylhydrazone
0	0·03	0	—
Pgl.	0·15	0·18	Trace
" + *M*/20 NaF	0·08	Trace	—
" + 0·2 mg. adenylic acid	0·57	1·80	+ +
" + 5·0 mg. creatine			
" + 5 mg. creatine	0·22	0·38	+

α-*Phosphoglycerol.* This ester is slowly dephosphorylated by normal calf cartilage but only at a *p*H higher than 7·6. No bisulphite-binding or hydrazone-forming substances could be detected as intermediary products of breakdown. There is no increase in lactic acid from this substance.

Pyrophosphoric acid. It may be of some interest to add that inorganic pyrophosphate is fairly rapidly converted by cartilage into orthophosphate. Additon of Mg has little effect upon the rate of this reaction.

Although it is evident that cartilage possesses enzymes which act upon a number of phosphoric acid esters, it appears to lack the substances which it attacks so readily. Trichloroacetic acid extracts were prepared from fresh cartilage and phosphate estimations were made after acid hydrolysis (*N* HCl, 100°) at 0, 7 and 30 min., 2 and 5 hr. as well as of the total acid-soluble P after ashing. However, there was no increase in phosphate content after acid hydrolysis or incineration.

That there are no substrates present in cartilage which can easily yield inorganic phosphate is suggested by the fact that the amount of phosphate which appears in aqueous cartilage extracts even on prolonged autolysis of the tissue at 37° is comparatively small (about 10 mg./100 ml.). Addition of toluene somewhat increases the amount of phosphate diffusing.

It seems also that cartilage lacks substances which would give rise to rapid ammonia formation, since not more than 0·01 mg. NH_3-N per g. is found after several days' autolysis.

For comparison with cartilage other joint tissues were examined with regard to their behaviour towards some of the phosphorylated compounds. Synovial fluid shows a content of 6–8 mg./100 ml. acid-soluble P and no acid-hydrolysable fractions are found. It does, however, decompose adenylic acid both by dephosphorylation and deamination, though more slowly than cartilage does. Here too, hypoxanthine is the end product. Hexosediphosphate undergoes dephosphorylation in synovial fluid with the formation of a bisulphite-binding substance and an orange 2:4-dinitrophenylhydrazone (Table 1). On the other hand, ligaments and synovial membrane, while showing a certain anatomical differentiation in their acid-hydrolysable P-fractions, appear to be inactive towards the substances mentioned above.

3. *Non-phosphorylated sugar-derivatives.* Before describing the results obtained with sugars a few remarks should be made with regard to the behaviour of lactic acid itself in cartilage. Fresh and well-rinsed cartilage shows a fairly high

content of lactic acid, 20–40 mg./100 g. (wet weight), which remains unchanged on incubation. A brief aqueous extraction of sliced cartilage brings practically the whole of the lactic acid into the fluid and on repeated extraction of the residue no more is obtained. Thus it would appear that washed cartilage has no ability to produce lactic acid. The lactic acid found in fresh cartilage is probably due to a post-mortem diffusion from blood and also perhaps from synovial fluid which was found to contain between 50 and 70 mg./100 ml. lactic acid. Other joint tissues, tendons and mucosa also contain some 20–30 mg. lactic acid/100 g. (wet weight).

Lactate is quite stable when added to cartilage alone but small amounts of it disappear in presence of cozymase, and at the same time traces of pyruvic acid can be detected (Table 3).

Glycogen. It has been known for a long time that cartilage contains a certain amount of glycogen. This might of course form a source of lactic acid under suitable conditions. The following experiment suggests that cartilage glycogen undergoes little change even during prolonged autolysis. 40 g. fresh articular cartilage were divided into two equal lots; in one of these glycogen was estimated at once, and the other was allowed to stand for a week at 20° with a few volumes of water and some toluene. Then the supernatant fluid was removed and the glycogen estimation carried out on the residue. After the acid hydrolysis of glycogen 130 mg. glucose/100 ml. were found in the fresh and 98 mg. glucose/100 ml. in the autolysed sample. The glycogen isolated from cartilage gives a strong red colour with iodine, it is precipitated with alcohol from acid solutions and rapidly fermented by Lebedew juice.

The estimation of the carbohydrate content in a cartilage extract (1 week old) shows 14 mg. sugar/100 ml., of which however only one third is dialysable. Some of the carbohydrate found in the extract may be due to small amounts of glycogen and chondroitin sulphuric acid and the rest to diastatic breakdown products of glycogen; for it was found that cartilage, as well as synovial fluid, possesses weak diastase activity towards starch and glycogen.

When, however, added glycogen is incubated with whole cartilage or cartilage extract in the presence of cozymase and adenylic acid small quantities of lactic acid are produced in almost all experiments (Table 3). Nicotinic acid amide cannot replace cozymase. In a few instances in addition to lactic acid small amounts of a bisulphite-binding substance could be detected and a slight precipitate of yellow 2:4-dinitrophenylhydrazone was formed, which was soluble with a red colour in KOH and probably due to pyruvic acid.

Table 3. *Lactic acid formation in cartilage*

10 mg. substrate. 2 g. cartilage. *p*H 7·6. Incub. 8 hr. 37°.

Additions	mg. lactic acid	B.B.S. in ml. *M*/100 I_2	2:4-Dinitrophenylhydrazone
0	0·38	0	—
Glycogen	0·78	0	—
Glycogen + cozymase + adenylic acid	1·98	0·22	Trace
Glucose	4·18	0	—
Galactose	0·90	0	—
Cori ester	1·16	0	—
Neuberg ester	1·00	0	—
Harden & Young ester + pyruvate + cozymase	5·28	19·2	+ + +
Pyruvate	0·58	22·3	+ + +
Pyruvate + cozymase	1·08	19·8	+ + +
Lactate	10·10	0	—
Lactate + cozymase	8·98	0·38	+

Hexoses. With other non-phosphorylated sugars, glucose, galactose, fructose, mannose and glucosamine, only small positive results were obtained even in presence of cozymase. There were however a few experiments with glucose where a significant increase in the lactic acid content took place (Table 3). No bisulphite-binding substances or hydrazone formation were observed.

Trioses. Of the three trioses examined, *dl*-glyceraldehyde, dihydroxyacetone and methylglyoxal, the first two remain practically unchanged under the influence of cartilage, whereas methylglyoxal disappears fairly rapidly both in aerobic and anaerobic conditions (Table 4). The reaction is thermolabile. The disappearance of methylglyoxal is due to its conversion into lactic acid. The course of the reaction can be followed by manometric measurements of CO_2-liberation from bicarbonate in equilibrium with 5 % CO_2 in N_2. Acid production in cartilage alone is small and varies between 30 and 50 μl. per g. in 4 hr.

The enzyme glyoxalase is known to convert methylglyoxal into lactic acid in presence of traces of GSH [Lohmann, 1932]. In cartilage (and in synovial fluid) the nitroprusside test is negative and yet the reaction proceeds quite well without added GSH. Addition of GSH to whole cartilage only accelerates the rate of the reaction to a moderate extent, but when cartilage extract is used as the source of enzyme the effect is more pronounced. In synovial fluid there is also a slow acid production from methylglyoxal. The effect of iodoacetate, to which the glyoxalase of other tissues is very sensitive, is comparatively small in cartilage (Table 4).

From a series of chemical estimations of the methylglyoxal used up and lactic acid formed, as well as from manometric data, it follows that somewhat more methylglyoxal disappears than is found as lactic acid, especially in anaerobic conditions. This would suggest that some other substance besides lactic acid is formed in the course of the reaction, but so far no direct evidence has been obtained (Table 4). Phenylglyoxal also gives rise to production of acid in cartilage (presumably mandelic acid). It may be mentioned that the ability of cartilage to act on methylglyoxal varies and in one case it was found completely inactive both aerobically and anaerobically.

Table 4. *Behaviour of trioses in cartilage*

5 mg. substrate. 1·5 g. cartilage. Incub. 3 hr. 37°.

Additions	mg. triose used up	mg. lactic acid formed	μl. CO_2-prod. (measured manometr.)
Glyceraldehyde in 5 % CO_2-95 % N_2	0·08	0·40	35
Dihydroxyacetone ,,	0·10	0·28	30
Methylglyoxal ,,	4·00	3·80	860
,, +GSH ,,	4·40	4·80	990
,, +$M/50$ iodoacetate ,,	2·00	1·50	310
,, in air	3·15	2·90	Not measured

Pyruvic acid. Very little pyruvic acid disappears when added alone to cartilage. In presence of cozymase a small proportion is converted into lactic acid. The lactic acid formation increases on addition of hexosediphosphate (Table 3). Cocarboxylase appears to have no effect whatever upon the breakdown of pyruvate in cartilage. Incidentally it was found that after prolonged incubation with cartilage, cocarboxylase loses much of its coenzyme activity as tested by the method of Lohmann & Schuster [1937].

Yet another substance tested in this series of experiments was *d*-glucurone, which however appeared to remain unchanged after incubation with cartilage.

Oxidation and reduction systems in cartilage. There is very little O_2-uptake when fresh finely-minced cartilage is tested manometrically (1–2 μl. per g. per hr.). Addition of synovial fluid is without effect, but carriers like methylene blue and pyocyanine slightly increase the reaction. In anaerobic conditions there is a slow, thermolabile reduction of methylene blue, 1·0 ml. $M/2000$ being reduced by 1 g. of fresh cartilage at 37° in 1–2 hr. In a few instances when cartilage slices were left for a day or two in phosphate buffer without an antiseptic the time for the methylene blue reduction fell to 20 min. but this acceleration was found to be due to bacterial infection. No O_2-uptake or methylene blue reduction was obtained with cartilage extracts.

The following substances were examined as substrates for aerobic and anaerobic oxidation by cartilage.

Succinate	Pyruvate	Glutamate
Lactate	Glucose	Alanine
α-Phosphoglycerol	Glucosamine	Adrenaline
Malate	Aldehyde	3:4-Dihydroxyphenylalanine
Citrate	Alcohol	Catechol
Acetate	Hypoxanthine	
Formate	Urate	

The necessary coenzymes and carriers were added and the experiments were generally arranged so as to afford for every reaction optimum conditions as known from the study of other tissues. Very small positive results were found with lactate in presence of cozymase, glucose in presence of coenzyme II, and hypoxanthine, but otherwise negative results were obtained.

When fresh cartilage is used for the aerobic oxidation of dihydroxyphenylalanine there is no O_2-uptake and the solution of the amino-acid in contact with the tissue remains colourless, while the control solution without tissue takes up a certain amount of O_2 and becomes dark. When instead of fresh cartilage toluene-treated cartilage is used for the experiment, there is a slight uptake of O_2 and the tissue and the solution of dihydroxyphenylalanine both become dark. Adrenaline, but not catechol, shows a similar behaviour.

On spectroscopic examination of a thick layer of powdered cartilage no cytochrome spectrum is visible and only a faint trace of reduced cytochrome *c* bands can be seen on addition of hydrosulphite. Similar observations were made with ligaments and synovial fluid.

Ascorbic acid was estimated in fresh cartilage, synovial fluid and synovial mucosa but negative results were obtained. Glutathione appears to be absent from cartilage and synovial fluid, judging by the negative nitroprusside test, but in some parts of the synovial mucosa the test is slightly positive.

On examination for heavy metals in cartilage, 0·5 mg. Fe was found per 100 g. vacuum-dried cartilage. Mn and Cu are absent.

Discussion

The present study was carried out in an attempt to elucidate the mechanism of glycolysis and oxidation in cartilage. The work of Robison and his collaborators has contributed much to our knowledge of the action of bone phosphatase on various phosphorylated glycolysis products, but little information was available with regard to lactic acid production in normal articular cartilage from these or any other substances.

The study of fermentation in yeast and muscle has established its dependence on certain catalysts such as the adenine nucleotides and cozymase. It appears

that these substances are relatively unstable when acted upon by calf cartilage. It may be assumed, however, that conditions exist for their resynthesis, e.g. by the interaction of adenylic acid with phosphoglyceric acid (p. 522). An interesting point in the behaviour of the adenine derivatives in cartilage is their breakdown to hypoxanthine, in contrast to their behaviour in muscle, where the process does not proceed beyond inosinic acid.

The results obtained with various substrates show that lactic acid formation is possible in cartilage (Table 3). They also suggest two mechanisms: one, which appears to involve the presence of cozymase, is the oxido-reduction between hexosediphosphate and pyruvate, in a manner similar to the glycolytic cycle in muscle, and another is the conversion of methylglyoxal into lactic acid. Cozymase must be added in experiments with glycogen and hexosediphosphate to enable the reaction to proceed, but the reaction with methylglyoxal requires no addition of GSH to cartilage although it is definitely accelerated by traces of GSH. Methylglyoxal seems to represent a normal breakdown product of hexosediphosphate, but even with the lactic acid it accounts for not more than 30–40% of the carbohydrate dephosphorylated. According to rotation measurements carried out by Martland & Robison [1929] the main product of decomposition of hexosediphosphate by bone phosphatase is a mixture of aldoses and ketoses and not fructose itself.

This enquiry into the oxidative systems proves that normal cartilage is rather poor in cytochrome and reducing substances such as ascorbic acid and glutathione, although there is obviously enough of the latter to evoke the glyoxalase activity in cartilage. These findings agree with the inability of cartilage to oxidize any of the many substances tested to an appreciable extent.

Summary

An attempt was made to study in detail enzyme systems in cartilage concerned with glycolysis and other oxido-reductions. Cartilage from young normal animals (calf) was used in the form of slices or extracts. For comparison some experiments were carried out with other joint tissues, synovial fluid, synovial membrane and ligaments.

Adenosinetriphosphoric, muscle adenylic, yeast adenylic and inosinic acids and adenosine are attacked in cartilage by dephosphorylation and/or deamination. Hypoxanthine is the end-product of these reactions. Cozymase and Warburg's coenzyme lose their catalytic activities after prolonged incubation with cartilage. Cocarboxylase also is unstable under the influence of cartilage.

The ability of cartilage to produce lactic acid from various phosphorylated and non-phosphorylated carbohydrates was tested. The small positive results obtained with some of the substances investigated suggest two mechanisms of lactic acid production in cartilage, one of which depends upon the presence of cozymase while the other is catalysed by glyoxalase. As intermediary products methylglyoxal is formed from hexosediphosphoric acid and pyruvic acid from phosphoglyceric acid.

Articular cartilage appears to be almost completely unable to oxidize substances such as succinate, lactate, aldehyde and several other substances which are rapidly oxidized in other tissues.

This work was carried out with the assistance of a grant from the Empire Rheumatism Council. The Co-operative Society and Messrs Warrington & Son, of Cambridge, have very generously supplied the experimental slaughterhouse material.

The author is indebted to Dr Stephenson for help in the bacteriological examinations and to Dr Drury for encouragement and advice and finally to Prof Sir F. G. Hopkins for his kind interest in this work.

REFERENCES

Birch, Harris & Ray (1933). *Biochem. J.* **27**, 590.
Bywaters (1937). *Path. Bact.* **44**, 247.
Clift & Cook (1932). *Biochem. J.* **26**, 1788.
Fiske & Subbarow (1925). *J. biol. Chem.* **66**, 375.
Friedemann, Cotonio & Shaffer (1927). *J. biol. Chem.* **73**, 335.
Hagedorn & Jensen (1923). *Biochem. Z.* **135**, 46.
Lohmann (1932). *Biochem. Z.* **254**, 332.
—— & Jendrassik (1926). *Biochem. Z.* **178**, 418.
—— & Schuster (1937). *Biochem. Z.* **294**, 188.
Martland & Robison (1929). *Biochem. J.* **23**, 237.
Mendel & Goldscheider (1925). *Biochem. Z.* **164**, 164.
Parnas & Heller (1924). *Biochem. Z.* **152**, 5.
Robison (1923). *Biochem. J.* **17**, 286.
Tillmans & Philippi (1929). *Biochem. Z.* **215**, 36.

63. STUDIES IN DETOXICATION

6. ETHEREAL SULPHATE FORMATION IN THE RABBIT AFTER ADMINISTRATION OF SULPHANILAMIDE

By JEAN SHELSWELL[1] and R. TECWYN WILLIAMS

From the Physiology Department, Medical School, Hospitals Centre, Birmingham

(*Received 19 February 1940*)

It has been shown that 50–90 % of an administered dose of sulphanilamide (*p*-aminobenzenesulphonamide) can be accounted for by the free and acetylated drug excreted in the urine [Marshall, 1939]. Some of it, however, undergoes oxidation *in vivo* and it has been suggested that *p*-aminophenol and *p*-hydroxylaminobenzenesulphonamide are products of this oxidation and may be responsible for some of the toxic symptoms [Brownlee, 1939; Rimington & Hemmings, 1939; Campbell & Morgan, 1939; James, 1939].[2] It appeared to us that if any phenolic substances were produced *in vivo*, then an increase in urinary ethereal sulphate should result. The phenolic hydroxyl appears to be the only group which conjugates with sulphate in the animal body (but see Stekol & Cerecedo [1933] for ethereal sulphate formation from *iso*barbituric acid). An increase in ethereal sulphate in the urine after feeding sulphanilamide would therefore be strong evidence in favour of the presence of a phenolic substance.

In a recent paper James [1940] has shown that, in eight patients receiving sulphanilamide, the ratio of inorganic to ethereal sulphate fell below the normally accepted value of 10, whilst in four patients receiving sulphapyridine (M and B, 693) the ratio was almost normal. This was taken to indicate a rise in ethereal sulphate after sulphanilamide but not after sulphapyridine. In view of this paper, we have decided to publish results which we had already obtained with rabbits, although our investigations are not complete since they form part of a wider research on the oxidation of sulphonamide drugs *in vivo*. We have found that on giving rabbits (under standard conditions) doses of sulphanilamide varying from 0·2 to 1·0 g./kg. an increase of ethereal sulphate is produced which is roughly proportional to the dose of the drug (see Table 2 and Fig. 1). Assuming that one phenolic hydroxyl is produced from one molecule of sulphanilamide, ethereal sulphate corresponding to 6–12 % of the sulphanilamide fed appears in the urine. We have avoided using the ratio inorganic sulphate to ethereal sulphate since we consider it unreliable as an indication of ethereal sulphate formation in the rabbit. We prefer to measure the increase of ethereal sulphate above the normal value under the standard conditions of diet etc., used in our experiments.

We have been unable to detect *p*-aminophenol or *p*-hydroxylaminobenzenesulphonamide in the urine of rabbits receiving sulphanilamide, although James [1939] has detected these substances in the urine of mice similarly treated.

[1] Caroline Harrold Research Scholar, University of Birmingham.

[2] Smith [1939] states that *p*-aminophenol produces less abnormal blood pigment that sulphanilamide.

Rabbit urines (hydrolysed and unhydrolysed) after sulphanilamide were also subjected to electrodialysis[1] but *p*-aminophenol could not be detected in the dialysates even though the merest trace of added *p*-aminophenol could be easily recovered and detected. We have found that sulphanilamide (but not sulphapyridine) gives the indophenol reaction unless the latter is very carefully controlled. It is interesting to note that the indophenol reaction for *p*-aminophenol is given by 1-hydroxy-2-aminobenzene-4-sulphonic acid (B.D.H.). Our investigations on the points mentioned in this paragraph are not yet complete and a full account of these reactions and of a glucuronide occurring in urines from rabbits treated with sulphanilamide will be published at a later date.

In four experiments carried out with sulphapyridine (M and B, 693), we have found a slight rise in ethereal sulphate in two and no detectable increase in the others.

Experimental

All sulphate values are expressed as SO_3 and the rabbits used were females. The general procedure, diet, conditions and methods of analysis were the same as described in an earlier paper [Williams, 1938]. Sulphanilamide (B.D.H., M.P. 165°) was administered in suspension in water by stomach tube. The water given was measured from a calibrated wash-bottle and was usually about 50–60 ml. Urine was collected and analysed every 2 days, the drug being administered on the third day of the experiment as indicated in the following example. No sulphanilamide was detected in the urine collected on the 5th–6th days of each experiment.

Exp. 17. Rabbit 66. Weight 2·2 kg. Given 2·2 g. sulphanilamide with 80 ml. water on the 3rd day.

Days	Urine vol.* ml.	Inorganic SO_3 mg.	Ethereal SO_3 mg.
1, 2	158	463	43
3, 4	232	705	102
5, 6	137	683	38·5

* Funnels were washed with water from a calibrated wash-bottle whereby the true urine vol. could be determined. Average normal SO_3 = 40·7 mg.; increase = 61·3 mg. equivalent to 131 mg. of sulphanilamide or 6 % of the dose fed.

Table 1 shows that during the hydrolysis with acid, which is necessary for estimating total sulphate (Folin) in urine, sulphanilamide does not produce extra sulphate.

Table 1

Exp.	Substance added to urine	Total sulphate obtained from 25 ml. urine in mg. $BaSO_4$
1*a*	—	241·6
1*b*	100 mg. sulphanilamide	243·4
2*a*	—	220·3
2*b*	100 mg. sulphanilamide	219·8

[1] The electrodialysis experiments were carried out for us by Dr W. V. Thorpe. A full account will be given in a later publication.

The results of the experiments are given in Table 2 and Fig. 1 shows the ethereal SO_3 increase/kg. of rabbit plotted against the dose of sulphanilamide per kg.

Table 2. *Formation of ethereal sulphate in the rabbit after various doses of sulphanilamide*

Rabbit no.	Rabbit wt. kg.	*A* Dose g.	Dose/kg.	Increase in ethereal SO_3 mg.	*B* Sulphanilamide equivalent to increase mg.	*B* as % of *A*
50	2·4	0·506	0·21	16·2	34·8	6·9
62	2·1	0·51	0·24	26·6	57·1	11·2
60	2·9	1·0	0·34	46·4	99·7	10·0
50	2·5	0·99	0·38	27·2	58·4	5·9
61	1·6	0·648	0·4	37·4	80·4	12·4
56	2·2	1·0	0·45	33·2	71·3	7·1
61	1·95	0·975	0·5	24·4	52·5	5·4
65	1·9	1·19	0·6	75·6	163·5	13·7
62	2·2	1·31	0·6	46·7	100·4	7·7
50	2·7	1·89	0·7	45·0	96·7	5·1
66	2·0	1·6	0·8	41·3	89·1	5·6
50	2·5	2·0	0·8	57·4	123·4	6·2
61	1·82	1·82	1·0	76·6	164·6	9·0
55	2·0	2·0	1·0	111·9	240·5	12·0
66	2·2	2·2	1·0	61·1	131·1	6·0
62	2·45	2·45	1·0	139·7	306·3	12·2

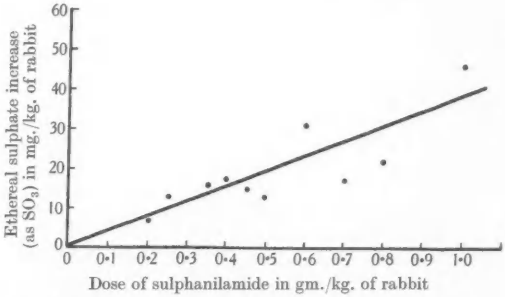


Fig. 1. Graph showing the increase of ethereal sulphate in the rabbit following the administration of varying doses of sulphanilamide.

Acetylsulphanilamide was obtained from all urines (30 experiments) except two, as a crystalline deposit mixed with phosphates. This deposit was filtered or centrifuged from the urine and recrystallized from hot water. About 25 % of the dose (1–3 g.) of sulphanilamide could be recovered in this manner. After three recrystallizations from water, the *p*-acetaminobenzenesulphonamide was obtained as long needles, M.P. 215° (Marshall [1937] gives M.P. 218°).

It was noted that, in many cases (but not all), sulphanilamide caused a diuresis; the effect, however, was variable. Braunstein & Parschin [1931] have shown that phenol excretion and conjugation may depend on urine volume, changes in the latter being due to the influence of the diet on water regulation. In view of this work it was therefore possible that the water given during sulphanilamide administration might influence the ethereal sulphate output, although Braunstein & Parschin's work showed that the larger the urine volume the less conjugated phenol is excreted. Experiments were therefore carried out

in which the rabbit received the same amount of water during the control periods as during administration of sulphanilamide. The following protocol shows the results of a typical experiment of this type. Water administration had no effect on the ethereal sulphate excretion.

Exp. 13. Rabbit 62. Weight 2·2 kg.

Days	Substance fed	Urine vol. ml.	Inorganic SO_3 mg.	Ethereal SO_3 mg.
1, 2	50 ml. water	178	452	37
3, 4	50 ml. water + 1·31 g. sulphanilamide	198	368	91
5, 6	50 ml. water	174	538	52

Average normal ethereal SO_3 = 44·5 mg.; increase = 46·5 mg. equivalent to 100 mg. sulphanilamide or 7·7% of the dose.

Neither did water appreciably influence the value of the normal ethereal sulphate, for in one experiment (three carried out) a rabbit excreted 45 mg. SO_3/2 days on the standard diet, whilst after administration of 50 ml. of water (+ standard diet) it excreted 41 mg. SO_3/2 days. Administration of 50 ml. of water to a 2–3 kg. rabbit had very little influence on the urine volume.

Summary

When sulphanilamide is fed to rabbits, the ethereal sulphate output in the urine is increased. The increase in ethereal sulphate is proportional to the dose of drug (0·2–1·0 g./kg.) fed. Assuming that one molecule of the conjugated sulphate is formed from one molecule of sulphanilamide, then 6–12% of the dose of drug gives rise to a hydroxy body, probably a phenol, which is excreted combined with sulphate.

We wish to thank Dr W. V. Thorpe for his interest in this work.

References

Braunstein & Parschin (1931). *Biochem. Z.* **235**, 344.
Brownlee (1939). *Biochem. J.* **33**, 697.
Campbell & Morgan (1939). *Lancet*, **237**, 123.
James (1939). *Biochem. J.* **33**, 1688.
—— (1940). *Lancet*, **238**, 25.
Marshall (1937). *Science*, **85**, 202.
—— (1939). *Physiol. Rev.* **19**, 251.
Rimington & Hemmings (1939). *Biochem. J.* **33**, 960.
Smith (1939). *J. Pharmacol.* **66**, 33.
Stekol & Cerecedo (1933). *J. biol. Chem.* **100**, 653.
Williams (1938). *Biochem. J.* **32**, 878.

64. RATE OF REJUVENATION OF THE SKELETON

By G. CH. HEVESY, H. B. LEVI and O. H. REBBE
From the Institute of Theoretical Physics, University of Copenhagen

(*Received 23 February 1940*)

The first experiments in which labelled (radioactive) phosphorus ^{32}P was applied as an indicator [Chievitz & Hevesy, 1935], showed that some of the phosphorus atoms of the mineral constituents of the bone exchange rapidly with those present in the plasma. This result was corroborated and extended by later work on this subject [Hevesy *et al.* 1937; Cook *et al.* 1937; Dols *et al.* 1937; 1939; Artom *et al.* 1938; Cohn & Greenberg, 1939; Lefevre & Bale, 1939]. The question as to what extent the P contents of the mineral constituents of the bone are replaced in a given time by plasma P remained unanswered however. This is an important question, as the rate of this replacement is a measure of the rate of rejuvenation of bone tissue. The extent to which bone P is replaced by plasma P in the course of a given time can be determined by comparing the activity of 1 mg. of bone P with that of 1 mg. of inorganic plasma P. The activity of the plasma P, i.e. its ^{32}P content, changes appreciably, however, with time. The ^{32}P atoms, like all P atoms present at any moment in the plasma, exchange with the P atoms present in the various organs and in doing so are removed from the plasma and replaced by tissue P. The application of the above-mentioned consideration implies a constancy of the activity of the plasma inorganic P. To secure such a constancy, we administered labelled phosphate all through the experiment, instead of doing so at the start of the experiment, as in all investigations mentioned above. By taking blood samples at intervals, we ascertained that the activity of the inorganic P of the plasma remained constant. At the end of the experiment, the bone sample was purified from all non-mineral constituents and the radioactivity of 1 mg. of bone P compared with that of 1 mg. of plasma inorganic P.

Experiments with frogs

Experiments of 5 to 240 min. duration were carried out with frogs. As early as 5 min. after injecting 0·3 ml. physiological NaCl solution containing a negligible proportion of labelled sodium phosphate into the lymph sac, the mineral constituents of the tibia contained some labelled phosphate, as shown in Table 1.

Table 1. *Labelled P contents of plasma and tibia of a frog*

Wt. 45 g.; temperature 22°; determinations 5 min. after the start of the experiment.

Fraction	^{32}P content per mg. P (specific activity)
Plasma	100
Epiphysis	0·026
Diaphysis	0·013

With increasing time, the ^{32}P content of the mineral constituents of the tibia increases (Fig. 1). After the lapse of 1 hr., the specific activity of the epiphysis P amounts only to 1/600 of the corresponding magnitude of the inorganic P of the

plasma. Thus only 1/600 of the epiphysial P was replaced by plasma P within 1 hr. In the next 3 hr. a further 1/900 part of the epiphysis P exchanged. The first point in the curve was obtained by analysing the right, the second point by analysing the left tibia. For the diaphysis, the corresponding figures were found to be 1/900 and 1/1200, respectively. In the course of 4 hr., therefore, only a minute part of the tibia P is replaced by plasma P. A still smaller replacement is found when the frog is kept at 0°.

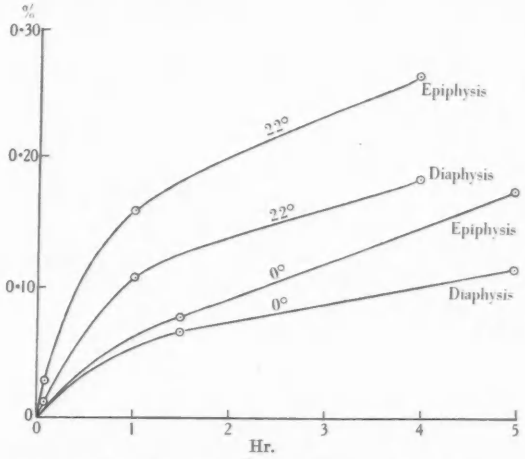


Fig. 1. Extent of replacement of frog's bone P by labelled P.

Experiments with rabbits

After the lapse of 2 hr., 1/530 and 1/1800, respectively, of the tibia epiphysis P and diaphysis P were found to be replaced by plasma P. With increasing time, an increase of the replacement of the bone P takes place, as shown in Fig. 2 and Table 2; this increase diminishes, however, with increasing time, as would be expected. The bone tissue contains numerous small crystals formed by mineralization of the matrix. The crystals are built up on similar lines to the mineral apatite.[1] While the atoms situated on the uppermost layer of the crystals [Paneth, 1922] exchange easily with those present in the surrounding liquid, those situated inside the crystal are prevented from doing so, except at very high temperatures. The exchange between bone phosphate and plasma phosphate which we observe in experiments of short duration is due to a replacement process between the phosphate ions situated on the surface of the apatite crystals and those of the plasma or lymph. Should the surface exchange be exhausted, an increase of the time of the experiment may at first have no effect at all on the extent of replacement. If, however, in the course of time a dissolution and reprecipitation of the apatite crystals takes place, new and far-reaching possibilities

[1] From X-ray measurements, it was concluded [Caglioti, 1936] that the inorganic part of the bone has the composition of about $3Ca_3(PO_4)_2.CaCO_3.xH_2O$ with the hexagonal structure of hydroxyapatite, the length of the axes being $a = 9{\cdot}2 \times 10^{-8}$ cm. and $c = 6{\cdot}9 \times 10^{-8}$ cm., the axis being oriented parallel to the length of the bone. The organic parts consist of polypeptide chains, supported and stretched.

of an exchange between plasma P and bone P will arise. From these considerations it follows that an exchange taking place within a long interval cannot be extrapolated from results obtained in experiments of short duration. We have, therefore, carried out experiments in which we kept the activity of the plasma inorganic P of rabbits at a constant level for several days or weeks.

Table 2. *Extent of replacement of the bone P of the rabbit by labelled P*

The labelled phosphate was injected intravenously during the experiment.

Fraction	2 hr. %	4 hr. %
Epiphysis	0·180	0·200
Diaphysis	0·056	0·106

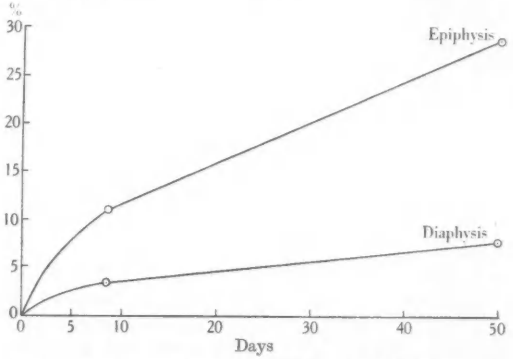


Fig. 2. Extent of replacement of rabbit's bone P by labelled P.

Experiment of long duration

To obtain a constant level of the plasma inorganic P, the first day every 30 min. and later twice every day, labelled sodium phosphate of negligible weight was administered by subcutaneous injection to rabbits. After removal of the marrow, the bone was first extracted for 12 hr. with hot ether-alcohol. The bone was then treated with hot alkaline glycerol solution for further 6 hr. The fractions obtained were dissolved in HNO_3 and their P contents precipitated as molybdate. The molybdate was dissolved in dilute NH_3 and precipitated as ammonium magnesium salt. An aliquot of the sample obtained was used in the colorimetric P determination, while another aliquot was reprecipitated as ammonium magnesium phosphate and its radioactivity measured with a Geiger counter. In prolonged experiments the analysis of the plasma inorganic P is conveniently replaced by that of the urine P. In Tables 3 and 4 the specific activities of the different bone P fractions are recorded.

Table 3. *Extent of rejuvenation of the tibia in the course of 9 days*

Wt. of rabbit: 2 kg.

Fraction	% P rejuvenated (specific activity)
Epiphysis P	11·2
Diaphysis P	3·2
Tibia phosphatide P	74·8
Marrow phosphatide P	80·1

In the course of 9 days, therefore, only 11 % of the epiphysis and 3 % of the diaphysis are rejuvenated, while most of the phosphatide molecules present in the marrow and in the bone are newly formed.

Table 4. *Extent of rejuvenation of the skeleton of a rabbit in the course of 50 days*

Fraction	% P rejuvenated
Femur epiphysis inorganic P	29·7
Femur epiphysis phosphatide P	100
Femur epiphysis glycerol extract* P	51·0
Femur diaphysis inorganic P	6·7
Femur diaphysis phosphatide P	*ca.* 100
Femur diaphysis glycerol extract* P	84·5
Tibia epiphysis inorganic P	28·6
Tibia diaphysis inorganic P	7·6
Costa	27·5
Scapula	43·8
Incisor dentine, apical	103
Incisor dentine, medial	98·5
Incisor dentine, incisal	41·2
Incisor enamel, apical + medial	82·0
Incisor enamel, incisal	6·6

* This fraction presumably contains some mineral P.

In the course of 50 days, only 29 % of the epiphysial and 7 % of the diaphysial mineral constituents were replaced (Table 4). The tibia and femur show about the same behaviour. About half of the scapula remained unchanged. The almost complete replacement of the apical and medial parts of the incisor dentine P can hardly be interpreted as due to an exchange between dentine P and plasma P, since the replacement rate of the dentine P was found to be lower than that of the tibia P [Hevesy *et al.* 1937; Lefevre & Bale, 1939] and since the tibia P, as seen above, was replaced only to a restricted extent. The high ^{32}P content of the incisor dentine must be due to an actual growth, to a formation by a calcification process. As a plasma containing ^{32}P was instrumental in calcifying the newly grown parts of the incisor, the P of the latter was bound to have the same specific activity as shown by the plasma P. As seen in Table 4, the P of the apical part of the incisor dentine investigated has, within the errors of experiment (± 5 %), the same specific activity as that of the plasma P. This part, having a length of about 0·9 mm., is entirely newly formed during the experiment. The bulk of the medial part of the dentine was freshly grown as well, while the incisal part, having a length of 1·2 mm., is only partially newly formed with participation of the labelled plasma. About half of the P atoms present in the incisal part of the dentine were not replaced; they must thus be those which were located in the apical or medial region of the incisor before the start of the experiment. The tissue containing these atoms was pushed forward *in toto*. Partly before this "slipping" process and partly during it, some of the P atoms of the dentine have the opportunity to exchange with labelled P atoms and, therefore, the P of the incisal part of the dentine shows an activity which amounts to about 1/3 of the specific activity of the plasma P. We see here an interesting case of tissue formation in which macroscopic aggregates are "slipped" from one place to another *in toto*, experiencing only a restricted atomic or molecular replacement. This effect is much more clearly shown in the growth of the enamel.

The apical and medial parts of the enamel are formed by a calcification process from labelled plasma and, therefore, these parts of the enamel became strongly active. From the fact that the incisal part of the enamel is only slightly active, we have to conclude that this fraction is not formed in the course of the experiment through a calcification process. Its crystals were formed at an earlier date

from non-labelled plasma and the whole fraction "slipped" *in toto* during the course of the experiment from the position in which it was calcified into the place it took up at the end of the experiment. The incisal end of the dentine is probably to a large extent also formed by "slip" *in toto* of the medial parts, though this conclusion is not supported as clearly by the activity figures arrived at in the case of the enamel. A part of the incisal dentine P had an opportunity to exchange to an appreciable extent before the "slip" took place and also during that process. Enamel P exchanges only to a minute extent [Armstrong, 1940]. It is also of interest to note that the activity figures exclude the possibility that the incisal part of the enamel is formed by extensive calcification of the outer region of the dentine. In that case, the enamel could not be much less active than the corresponding dentine part. The foundation of the incisal enamel is laid in the apical end and reaches its final position without interchange with the dentine. As both the P and Ca of the teeth have their origin in the plasma, the application of labelled Ca as an indicator can be expected to lead to similar results to those found above.

Epiphysial and diaphysial bone tissues

The epiphysis was found to exchange the P content of its mineral constituents at a higher rate than the diaphysis, as seen in Table 5.

Table 5. *Ratio of the specific activities of the epiphysial and diaphysial P of the tibia of rabbits*

The level of the activity of the plasma inorganic P was kept constant all through the experiment.

Time	Ratio
2 hr.	3·2
4 „	1·9
10 days	3·5
50 „	3·8

In experiments with frogs, in which the labelled phosphate was injected into the lymph sac at the start of experiments, taking 1–22 days, the ratio 1·3–1·6 was found. In these experiments, the tibia and femur were both investigated and the average was taken. In an investigation of the tibia P of rats, to which the labelled phosphate was administered at the start of the experiment, the ratios 3·1, 2·9, 2·5, 1·7 and 1·8 were found after $\frac{1}{2}$ hr., 4, 10, 50 and 110 days, respectively. It is of interest to remark that from the finding that the diaphysial P is only about half as active as the epiphysial P 110 days after the start of the experiment, we can conclude that an appreciable part of the skeleton has not been renewed within 110 days.

In experiments on chickens, Dols *et al.* [1939] found the above ratio to be 3, 22 hr. after administration of labelled phosphate; rachitic chickens gave the ratio 2·5.

The more rapid exchange of the epiphysial P is just what would be expected. The epiphysis is characterized by a poorer mineralization of the matrix than is the diaphysis and contains more organic matter and water than the diaphysis. The circulating lymph, containing the labelled P, will therefore reach the apatite surface more easily in the first-mentioned case. Should the size of the apatite crystals be smaller in the epiphysis than in the diaphysis and therefore the ratio surface : volume be larger in this type of bone tissue, one would also expect a more rapid exchange of the mineral constituents. Whether such a difference in the size of the apatite crystals actually occurs is not known. X-ray

investigations [Bale *et al.* 1934] lead to the result that the size of the ultimate crystals of dentine and bone is about 10^{-6} cm., while the very effectively mineralized enamel contains larger crystals (10^{-5} cm.). It is, therefore, quite probable that the difference in the extents of mineralization of the epiphysis and diaphysis manifests itself in a moderate difference in the sizes of the ultimate crystals in the two types of bone tissue. It is of interest to note that the difference in hardness of the different types of bones is, to a large extent, the result of a different degree of orientation of the crystallites of the bone. X-ray patterns indicate that orientation occurs during growth and first in those bones where the need for solidity (e.g. leg bones) is greatest [Caglioti & Gigante, 1936].

Summary

Labelled (radioactive) phosphate was administered to rabbits and frogs repeatedly during the experiment in order to keep the radioactive plasma inorganic phosphorus at a constant activity level. The comparison of the activity of 1 mg. bone inorganic P with that of the plasma inorganic P permits us to conclude to what extent the mineral constituents of the bone were renewed during the experiment.

Within 50 days, 29% of the femur and tibia epiphysis were found to be renewed, while the corresponding figure for the diaphysis amounted to 7%. Only half of the mineral constituents of the scapula were found to be unchanged. The phosphatides of the bones and the marrow were entirely renewed.

The phosphorus of the apical part of the dentine of the rabbit's incisor was found to have the same activity as the plasma phosphorus. From this result it follows that this part of the dentine was grown with the participation of labelled plasma phosphorus during the experiment. The greater part of the incisal end of the dentine was not formed by a calcification process *in situ* but by a "slip" of the apical part of the dentine.

The same behaviour is shown even more pronouncedly by the enamel. No interaction of any significance takes place between the incisal part of the enamel and the dentine either during their formation or at a later date. The atoms present in the former are to a very large extent those which were previously located in the apical (medial) part of the enamel.

The authors wish to express their gratitude to Prof. Niels Bohr for putting numerous facilities at their disposal and to Prof. Ernest O. Lawrence for the gift of the active phosphorus.

REFERENCES

Armstrong (1940). *J. biol. Chem.* (in the Press).
Artom, Sarzana & Segrè (1938). *Arch. int. Physiol.* **47**, 245.
Bale, Hodge & Warren (1934). *Amer. J. Roentgenol.* **32**, 369.
Caglioti (1936). *Atti Congr. naz. Chim. pura appl.* **1**, 320.
—— & Gigante (1936). *R.C. Accad. Lincei, Classe sci. fis.* **23**, 878.
Chievitz & Hevesy (1935). *Nature, Lond.*, **136**, 754.
—— —— (1937). *Kgl. Danske Vidensk. Selsk. Biol. Medd.* **13**, 9.
Cohn & Greenberg (1939). *J. biol. Chem.* **128**, 116 and **130**, 625.
Cook, Scott & Abelson (1937). *Proc. nat. Acad. Sci.* **23**, 528.
Dols & Jansen (1937). *Proc. Acad. Sci. Amst.* **40**, 3.
—— —— Sizoo & Van der Maas (1939). *Proc. Acad. Sci. Amst.* **42**, 2.
Hevesy, Holst & Krogh (1937). *Kgl. Danske Vidensk. Selsk. Biol. Medd.* **13**, 1.
Lefevre & Bale (1939). *J. biol. Chem.* **129**, 125.
Paneth (1922). *Z. Elektrochem.* **28**, 113.

65. THE DETERMINATION OF VITAMIN D IN FOOD SUBSTANCES CONTAINING PHOSPHORUS

By KATHARINE HOPE COWARD and ELSIE WOODWARD KÁSSNER

From the College of the Pharmaceutical Society, London

(*Received 7 February 1940*)

The diet of the rats used in most determinations of vitamin D has a high-Ca, low-P content, the ratio Ca:P being 4:1 or as much as 8:1. These figures are always based on total P in spite of the fact that some of it may be in organic combination and not available [Bruce & Callow, 1934, 1]. It is now a matter of common knowledge that the rickets so produced can be largely, if not completely, healed by the addition of phosphates to bring the Ca:P ratio more nearly equal to 1. The absolute concentrations of Ca and P in the rachitogenic diets are rarely reported, and Shohl [1937] has in fact shown that in each of 8 different Ca:P ratios there were zones where rickets was produced when the absolute amounts of Ca and P were low, and other zones where no rickets was produced when the amounts were increased. The healing produced by changing the Ca and P content is apparently similar to that produced by giving vitamin D in addition to the rachitogenic diet. Hence the healing brought about by a dose of a food substance whose vitamin D content is being determined may be due partly to the vitamin D and partly to the P contained in the substance. This might be considered acceptable as a measure of the healing effect of this food substance if all cases of rickets were due to the high-Ca, low-P content of the diet consumed; but as rickets is also produced by a low-Ca, high-P diet, the giving of a food substance containing more P and even a certain amount of vitamin D also might do more harm than good. The determination of vitamin D in a food substance should therefore be a measure of this factor and of nothing else. Key & Morgan [1932] made an attempt to simplify determinations of vitamin D in food substances which contained enough P to alter the Ca : P ratio of the rachitogenic diet used by them. By replacing part of the calcium carbonate of the diet by the appropriate amounts of calcium phosphate they made diets with Ca : P ratios of 4:1, 4:2, 4:3 and 4:4. The effects of giving these diets to rats rendered rachitic by feeding on a diet with a Ca : P ratio of 4:1 were then compared with the effects of feeding graded doses of vitamin D, viz. 0·25, 0·5, 1·0 and 2·0 units daily, by simultaneous comparisons. From two experiments on rats with "slight" and "severe" rickets respectively, they concluded that changing the Ca : P ratio from 4:1 to 2:1 was equivalent to giving a dose of 0·7 unit of vitamin D daily. They concluded, however, that it was preferable to adjust the Ca : P ratio of the diets of the rats receiving the standard to that of the rats receiving the test substance rather than to make an arithmetical allowance in calculating the result, particularly as they had so little information as to the effect of other changes in the Ca : P ratio.

Bruce & Callow [1934, 2] pointed out that if the relation between healing and dose of vitamin D given was logarithmic when a diet with a particular Ca : P ratio was used and the effect of giving both extra P and vitamin D was an additive one, then the curve of response to graded doses of vitamin D plus a constant amount of P would not be logarithmic. By experiment they found that the

effect of altering the Ca : P ratio and at the same time giving vitamin D was indeed multiplicative and not additive. The curves of response relating healing to the logarithm of graded doses of vitamin D given, obtained with diets of different Ca : P ratios, were all straight lines and parallel to each other within the limits of experimental error. It seemed then desirable to test this point for ourselves with an arrangement of the experiment more like that of a determination of vitamin D in a foodstuff which might contain enough Ca and P in the dose tested to upset the Ca : P ratio of the basal diet. In two series of experiments we found that the response to a particular dose of vitamin D was not constant but that it bore a logarithmic relation to the dose of potassium phosphate given at the same time. Since this paper was written, O'Brien & Morgareidge [1939] have reported similar findings.

Experimental

We consider it essential in vitamin D determinations to divide each litter of rats equally between the groups given the one (or more) dose of standard and the one (or more) dose of test substance. The number of groups used and hence the number of doses tested at any one time are thereby limited. Therefore we carried out a number of separate experiments in each of which we compared the amounts of healing produced by:

(*a*) a certain dose of vitamin D (5 units in one series of experiments and 10 units in the other);

(*b*) a certain decrease in the ratio Ca : P made by adding potassium phosphate to the diet;

(*c*) the same dose of vitamin D plus the same decrease in the Ca : P ratio.

The comparisons were made by means of the "line test". For calculating changes in the Ca : P ratio, it was assumed that all the P in the rachitogenic diet was available.

In a preliminary experiment it was found that the rats would not take potassium phosphate as a separate dose even when mixed with dextrin and a little water. In all experiments reported in Table 1 therefore the amount that it

Table 1. *The healing brought about in rachitic rats by giving (a) 5 or 10 units of vitamin D, (b) graded doses of potassium phosphate and (c) 5 or 10 units of vitamin D plus graded doses of potassium phosphate, as judged by the "line test"*

No of rats	Amounts of K_2HPO_4 added to 70 g. diet, mg.	Ca : P ratio of diet	Average healing from (*a*) 10 units vit. D	(*b*) Additional P	(*c*) 10 units vit. D + added P	Difference between (*c*) and (*a*) healing
45	60	3·84:1	1·46	0·00	1·55	0·09
42	115	3·69:1	1·51	0·00	2·34	0·83
45	230	3·43:1	2·08	0·08	3·29	1·21
45	460	3·0:1	2·28	0·12	4·29	2·01
45	920	2·4:1	1·28	0·15	4·08	2·80
45	1380	2·0:1	1·38	0·88	4·87	3·49
			(*a*) 5 units vit. D	(*b*) Added P	(*c*) 5 units vit. D + added P	
45	460	3·0:1	0·94	0·13	2·85	1·91
42	920	2·4:1	0·74	0·53	2·86	2·12
45	1380	2·0:1	0·73	0·82	4·20	3·47

was desired to give each rat in 10 days was mixed with 70 g. of food so that there would be the same change in the Ca : P ratio of all rats on any particular dose, though there would not necessarily be the same absolute amounts eaten.

Results

The results of the experiments are summarized in Table 1. In every experiment the healing brought about by giving both vitamin D and additional P was greater than the sum of the healings brought about by the two treatments separately. Thus there was abundant evidence that the effect of giving both vitamin D and extra P was much more than additive. In particular it may be noted that in the series in which a dose of 10 units of vitamin D was always given, the lower doses of phosphate alone were not enough to bring about measurable healing but with the constant amount of vitamin D the healing was measurable and graded to the dose of phosphate given. Curves were obtained from these experiments by plotting average healing against:

(*a*) Graded doses of phosphate only, omitting lower doses which gave negligible healing (i) in comparison with 10 units vitamin D plus phosphate,

$$y = 4{\cdot}145\,x - 12{\cdot}136,$$

(ii) in comparison with 5 units vitamin D plus phosphate

$$y = 1{\cdot}647\,x - 4{\cdot}351.$$

(*b*) Graded doses of phosphate + 5 units vitamin D,

$$y = 2{\cdot}524\,x - 4{\cdot}076.$$

(*c*) Graded doses of phosphate + 10 units vitamin D,

$$y = 2{\cdot}324\,x - 2{\cdot}398.$$

The average slope of the two curves (*a*) (i) and (ii) is 2·896 which is very nearly equal to the slopes of the curves (*b*) 2·524 and (*c*) 2·324. Thus we have three curves of response to graded doses of phosphate given of equal slopes and at heights dependent upon the dose of vitamin D given at the same time.

For practical purposes this is as far as the calculation need be taken. It demonstrates very clearly that the vitamin D potency of a substance containing enough Ca and P in the dose tested to alter the Ca : P ratio of the rachitogenic diet cannot be determined directly by comparison with the standard. Moreover, it would be well-nigh impossible to construct a curve or table of sufficient accuracy to use for correcting the healing apparently due to vitamin D but partly due to changes in Ca : P ratio. Even if such a table were once constructed, the labour of determining the Ca : P ratio of the particular batch of diet used in any experiment and the amounts of Ca and P in the dose of test substance given, and especially of the amounts of available P in the two products, would be almost prohibitive. Even the labour of making these determinations on the test substance only, with a view to giving the appropriate amounts of salts of Ca and P to the control rats on the standard, would be heavy. The safest plan seems to be to extract the ether-soluble portion after saponification of the substance under examination and make a determination of the vitamin D content of the extract by direct comparison with the standard in the ordinary way.

A point brought out in the last column of Table 1 is worth considering. The figures in this column were obtained by subtracting the healing brought about by the dose of vitamin D only from the healing brought about by the dose of vitamin D plus the dose of phosphate. When the differences of the first series of experiments are plotted against the doses of phosphate given a curve of response is formed

whose equation is $y=2{\cdot}398 \log x-4{\cdot}242$. The slope of the curve is similar to the slopes of the curves of response to doses of vitamin D plus phosphate, which was to be expected, but the points lie more nearly on the line calculated than do the points of either of these other curves. This also would be expected since the variation in response between litters has been virtually eliminated by the subtraction of the healing produced by vitamin D only in each separate experiment. In fact for those workers who still doubt that there is a variation in response to vitamin D between different litters in addition to the variation in response between members of one litter, this finding should prove strong enough to remove their doubt. It is tempting to make calculations of the significance of the distances between the curves of response to 5 units plus phosphate and to 10 units plus phosphate respectively but that procedure cannot be sound because there was no "litter-mate" control between the different experiments.

Summary

A reinvestigation has been made of the effect of giving vitamin D plus phosphate to rats fed on a rachitogenic diet of high-Ca, low-P content. In six separate experiments the average healings produced by (*a*) 10 units of vitamin D, (*b*) a dose of potassium phosphate and (*c*) 10 units of vitamin D plus a dose of phosphate were compared. The dose of phosphate was the same in groups (*b*) and (*c*) of each experiment but ranged from 60 to 1380 mg. per rat approximately in the different experiments. A shorter series of three experiments was carried out with doses of (*a*) 5 units of vitamin D, (*b*) a dose of phosphate and (*c*) 5 units of vitamin D plus a dose of phosphate. In this series the doses of phosphate were 460, 920 and 1380 mg. respectively per rat.

The lower doses of phosphate alone were practically without effect on calcification but all doses affected the calcification brought about by either 5 or 10 units of vitamin D. The curves of response to (*a*) 10 units of vitamin D plus graded doses of phosphate, (*b*) 5 units of vitamin D plus graded doses of phosphate and (*c*) graded doses of phosphate alone (omitting those which were very nearly zero) were all logarithmic and of similar slope, though at different heights, the curve (*a*) being the highest and (*c*) the lowest.

Therefore, as shown by Bruce & Callow [1934, 1, 2] by a slightly different method, it has been determined that giving both vitamin D and phosphate to rats receiving a rachitogenic diet which has a high-Ca, low-P content has a multiplicative effect on healing, not an additive one.

The only practical method, therefore, of determining the vitamin D content of the food substance which contains enough P in the dose tested to alter the Ca : P ratio of the diet given is to extract the ether-soluble portion after saponification and determine the vitamin D content of the extract.

REFERENCES

Bruce & Callow (1934, 1). *Biochem. J.* **28**, 512.
—— —— (1934, 2). *Biochem. J.* **28**, 517.
Key & Morgan (1932). *Biochem. J.* **26**, 196.
O'Brien & Morgareidge (1939). *J. Nutrit.* **18**, 277.
Shohl (1937). *J. Nutrit.* **14**, 69.

66. INVESTIGATIONS INTO VITAMIN A-FREE BASAL DIETS. III

By ALFRED LOUIS BACHARACH

From the Biochemical Department, Glaxo Laboratories Ltd., Greenford, Middlesex

(*Received* 12 *February 1940*)

Some years ago a series of investigations was begun in these laboratories with the object of achieving as close an approximation as possible to the "ideal" basic diet for vitamin assay, and in particular for the assay of vitamin A by the "curative" procedure generally used. Two papers were published [Bacharach, 1933; Bacharach & Lester Smith, 1933] to describe our earlier results. In 1934 we began to introduce further modifications into our vitamin A-free basal diet; by 1936 we regularly employed a diet having the following composition:

	Diet F Parts	
Rice starch	50	Fed as a paste with water. Every animal receives also 0·375 μg. of calciferol per week
Arachis oil	10	
Casein (alcohol-extracted)	20	
Salt mixture*	5	
Dried yeast	5	
Yeast extract	2·5	
Wheat germ (fat-free)	2·5	

* No. 6, according to de Loureiro [1931].

This was found to give a more constant—and shorter—period of "running out", a higher incidence of xerophthalmia and, at the same time, a greater response to curative doses than the diets described in our two earlier papers, and has now been in continuous use since 1936.

The improvement in the diet is, in our opinion, due mainly to two causes. First, the constituents carrying vitamin B_1 and the vitamin B_2 complex have been increased from 5% in diets A and B [Bacharach, 1933] to 10% in diets C and F (2·5% yeast extract is included to increase general palatability and ease of mixing, although it is a poorer source of water-soluble vitamins than an equivalent weight of the solids of dried yeast). A modified diet F containing an extra 5% of dried yeast was found by experiment to be in no way superior to diet F, which we therefore consider to contain at least optimal amounts of the necessary water-soluble vitamins. Secondly, alcohol extraction removes traces of vitamin A from the casein without seriously affecting its biological value. Maitra & Moore [1939] have recently shown that "light-white" casein (that is, sodium caseinogenate) contains traces of vitamin A; it is almost certain that our casein contains similar traces before extraction with alcohol which is thus a more efficient method of removing such traces than is dry heating, besides being free from other objections.

We were interested to discover how far diet F approached the "ideal" laid down in our earlier paper. Would it, in fact, if supplemented with adequate vitamin A, permit young stock animals at least to grow normally, if not also to achieve normal fertility and a normal life-span?

Preliminary experiments suggested some residual inadequacy of diet F. The difference between animals receiving the diet with a (relatively) large supplement of vitamin A and those receiving our stock diet was shown in growth rates differing appreciably and probably significantly, though this difference was much less than was obtained in a comparison of the earlier diet C with the stock diet. Careful scrutiny of the constituents of diet F had failed to indicate any obvious dietary deficiency, when, for us, a most fortunate discussion of the whole question with Prof. A. Sordelli caused him to suggest an investigation into the relative palatabilities, as judged by the consumptions, of the two diets under apparently identical conditions. Although a little alarmed at the prospect, we decided to undertake the paired-feeding experiment which, it seemed to us, alone could settle the question.

Actually, "triad-feeding" was necessary, so as to have three strictly comparable groups of animals receiving, respectively, unlimited diet F (with carotene supplement), stock diet limited to the intake level of diet F and unlimited stock diet. The two diets had almost exactly equal calorific values. The animals on diet F received a daily ration of 20 μg. purified β-carotene. There were 12 males and 12 females in each group except the *ad libitum* stock diet group, for which only 11 females were available. In Table 1 the results have been given for the two sexes separately. In Fig. 1, combined growth curves are shown.

Table 1

	Vitamin A-free diet *ad lib.* + carotene		Stock diet limited		Stock diet *ad lib.*	
	♂	♀	♂	♀	♂	♀
Total food intake (g.) per animal in 100 days	1212	1065	1207	992	1384	1164
Average wt. increase (g.) in 100 days	210	129	216	133	238	135
S.D. of individual wt. increases	23·6	7·6	25·0	14·8	35·1	21·3

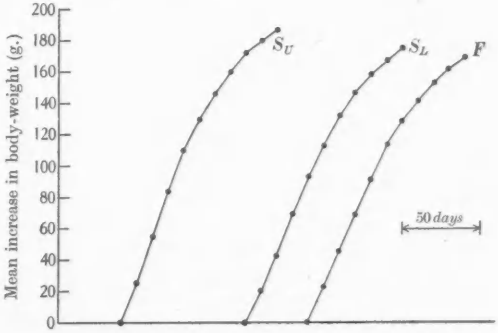


Fig. 1. Increase of body-weight in 100 days. S_U, mean values for 12♂ and 11♀ receiving stock diet F *ad lib.* S_L, mean values for 12♂ and 12♀ receiving stock diet, limited in amount to average intake of diet F. *F*, mean values for 12♂ and 12♀ receiving diet F *ad lib.*, with about 25 I.U. of β-carotene per animal per day.

The difference in mean weight-increases of male animals receiving unlimited or limited amounts of stock diet shows a degree of significance corresponding with a value of $P=0{\cdot}09$, barely sufficient to justify connecting the distinct

difference in food intakes with the apparent difference in growth-rates. No other even nearly significant differences are to be found between the effects of pairs of diets.

We concluded, therefore, that we were justified in considering diet F a good approximation to the "ideal" basal diet, differing mainly, if at all, in a somewhat lowered palatability, and a consequent slightly reduced intake, from our stock diet which is known to have been adequate for health and fertility over many generations.

Use of diet F in many vitamin A assays has led to fairly satisfactory results: as has been pointed out by several workers, particularly by Richards & Simpson [1934], the nature of the "curative" vitamin A test makes the use of sick animals almost inevitable, and thereby introduces an inevitable source of error and uncertainty. Any proposals to improve still further basal diets are closely scrutinized by all workers in the field, and we learned with interest of the advantages claimed by Tainsh & Wilkinson[1] [1939] for the replacement of part of the basal diet by its equivalent (in protein nitrogen) of coconut meal. We decided to test this claim and at the same time to take the opportunity of making certain other observations, especially of comparing the relative values of male and female animals of our stock under our experimental conditions as "reagents" for vitamin A, and also to obtain an estimate of the probable limits of error obtainable in assays carried out by us. The composition of the modified diet (diet G) was as follows:

	Diet G Parts	
Coconut meal	30	Calciferol supplement as with diet F
Rice starch	25	
Casein (alcohol-extracted)	15	
Arachis oil	10	
Salt mixture*	5	
Dried yeast	5	
Yeast extract	2·5	

* As in Diet F.

For reasons not germane to the nature of the experiment, the fat-free wheat germ (2·5 %) was omitted from this diet. This slightly reduced the intake of aneurin and of certain constituents of the vitamin B_2 complex, but any difference in effect of the two diets can only have been in very small measure due to such a change; this is shown by the behaviour of the negative controls, for which diet F actually appeared more drastically deficient than diet G (see below). 10 g. of either diet furnished between 15 and 30 μg. (5 to 10 I.U.) of aneurin per day; vitamin B_1 shortage is not, therefore, a limiting factor in either. The proximate analyses (calculated) of the two diets were:

	Diet F (%)	Diet G (%)
Fat	10·6	13·1
Protein	25·8	25·3
Carbohydrate	50·0	43·3
Ash	6·1	7·2
Fibre	—	3·3
Moisture	7·1	7·4
Calories per kg.	4085	4020

The number and distribution of the animals used in evaluating the results, as well as the assay figures obtained for the sample of oil and the probable limits of error of these figures, are given in Table 2. All the results were calculated by the

[1] We are much indebted to these authors for information about their results some months before publication.

Table 2

Diet	Sex	No. of animals	I.U. of vitamin A found per g. ($\bar{A}$)	σ_M*	b*	Probable limits of assay error† $P=0{\cdot}99$ %	$P=0{\cdot}99$ I.U. per g.	$P=0{\cdot}95$ %	$P=0{\cdot}95$ I.U. per g.
Routine	♂	48	770	0·0719	63·1	65·5–153	505–1185	72·5–138	560–1070
Modified		56	830	0·0912	34·6	58·0–172	485–1430	66·5–151	550–1250
Routine	♀	52	507	0·1230	25·3	48 –207·5	245–1050	57·5–174	290–885
Modified		56	510	0·4497	7·5	7 –1440	35–7340	13 –760	67–3880

* $M=\log \bar{A}-3$. † Calculated from the data of the experiment itself.
Response $=a+b$ log dose.

method of Irwin [1937]. The doses of oil fed (twice a week) were equivalent to daily doses of 1·84 and 3·67 mg., the doses of Standard to 2 and 4 I.U. Every set[1] of isogenic animals was represented by one animal in each dosage group and in some instances by a negative control. The period of test was 5 weeks. Only those sets of isogenic animals in which all 4 dosed animals survived the test period without loss in weight have been included for purposes of calculation. Actually there were 5 males that died on the routine diet F, and 2 females that lost weight, but survived; the corresponding figures for the modified diet were 2 males, and 1 male and 1 female. One set of females on the modified diet has been omitted because of an accidental injury to one animal in the set.

Tests for significance have revealed certain points that bear on the questions raised earlier in this paper. The error of each of the 4 assays—taking the 2 diets and the 2 sexes separately—has been calculated from the responses obtained, according to the method of Irwin [1937]. No one of the assay values differs significantly from any of the others, though clearly those obtained with male rats are more trustworthy than those obtained with females. Further, the introduction of coconut meal into diet F and its transformation thereby into modified diet G has been accompanied by a marked lowering of its utility, especially when female rats are being used. It has, in fact, made females useless for the assay. Our finding as to the relative superiority of bucks over does for vitamin A assay—and the explanation of this superiority—is in good agreement, in spite of our different attack on the problem, with that of Coward [1932]. We have also compared our results with the findings of Coward [1933] as to the effect of the length of a test on its accuracy. We have calculated the values obtained with male rats on our routine diet for the 3-week, as well as for the 5-week, period and have found (Table 3) no increase in accuracy as the result of prolonging the test. In this respect, we differ slightly from Coward [1933] who obtained an increase in accuracy with the longer test period, but not sufficient to make worth while the extra work involved.

More detailed information about the responses given by the various groups of animals is summarized in Table 4, where the effects of modifying the diet and of the sex of the experimental animals are shown on the standard deviations of the responses and on the slopes of the various contributory response curves as well as on the standard deviations of these slopes. The lower errors, are, in part, associated with lower mean responses, but their correlation is found not to be significant; calculation of the coefficients of variation (the standard deviations expressed as percentages of the mean responses) shows no evidence of any trend.

[1] It is suggested that, to avoid a source of confusion from which the literature is by no means free, the word "group" be strictly confined in this kind of experiment to animals receiving the same dose, while the terms "litter", "set", "series" etc., as applicable, be used for the collection of individual animals over which the different doses are distributed.

Table 3

Length of test weeks	Value of b	Result of assay: I.U. per g. = antilog ($M+3$)	σ_M	Probable limits of error: $P=0{\cdot}95$	$P=0{\cdot}99$
3	42·6	814	0·0716	72·5–138	65·5–153
5	63·1	772	0·0719	72·5–138	65·5–153

Table 4

Diet	No. and sex of animals on each dose	Dose	Weight increases (5 weeks): Mean increase, Vitamin A	Mean increase, Carotene	Standard deviation of increase (σ_W), Vitamin A	Standard deviation of increase (σ_W), Carotene	Slope of contributory response curves, b (with standard errors, σ_b), Vitamin A	Slope of contributory response curves, b (with standard errors, σ_b), Carotene
Routine	12 ♂	Upper	58·5	68·3	14·8	16·8	61·7	64·5
		Lower	39·9	48·9	18·1	15·7	(22·7)	(21·9)
Modified	14 ♂	Upper	54·7	57·8	10·9	14·8	44·9	34·3
		Lower	41·2	47·4	11·6	18·7	(14·8)	(21·3)
Routine	13 ♀	Upper	41·7	46·6	5·25	7·4	38·8	13·7
		Lower	30·5	42·5	7·2	9·7	(8·3)	(11·4)
Modified	14 ♀	Upper	38·2	39·2	17·0	9·4	12·3	2·6
		Lower	34·5	38·4	8·8	11·8	(17·2)	(13·2)

The females on the routine diet showed a coefficient of 19%, the males on the modified diet 29% and both the other two groups of animals 32%; the coefficient was higher for the lower doses and slightly lower for the routine diet than for the modified diet.

Table 4 makes clear, however, that the main effect of sex and diet modification has been on the values of b, the slopes of the contributory and mean response curves. The "discrimination" shown by the animals—that is to say, the extent of the difference in response to a given difference in doses—was greater for males than for females, and greater on the routine diet than on the modified diet. In 3 out of 4 instances, males on the modified diet and females on either diet, the value of b was higher for vitamin A than for carotene. In this respect our findings appear to differ from those of Mead *et al.* [1939], who obtained no such difference under the conditions of their experiments, but to confirm the results of Tainsh & Wilkinson, quoted in those authors' paper. When, however, the standard errors of b are calculated for all the relevant pairs of comparisons, we find that the values of b only differ significantly for males and females. The difference for the values on routine and modified diets just fails to reach the 5% level of significance; the difference for test substance and carotene is not significant.

Females on the modified diet failed to discriminate significantly between the two doses (in the ratio of 2 : 1) either of vitamin A ($P=0{\cdot}18$) or of carotene ($P=0{\cdot}75$) and females on the routine diet showed discrimination of doubtful significance ($P=0{\cdot}07$) between the two doses of carotene. Other authors have noted the reduction in the error of vitamin A assays to be obtained by using only males; thus Coward *et al.* [1931] point out that the value of b, the slope of the response curve, is greater for males than for females, so that the value of σ_M, the standard deviation of the logarithm of the result of an assay, must be greater with females than with males, provided, of course, that a lower standard deviation of response by females does not set off the difference in slope. Our figures confirm Coward's finding that the standard deviation of response (increase in body-weight) is less for females than for males; the values for the mean standard

deviation (weighted) are, for males 17·3 and for females 10·7. Thus our results indicate that, even with the basal diets giving the higher values of b, the difference in utility between males and females is mainly due to their difference in "discrimination", any difference in the values for the standard deviations of the responses being insufficient to compensate for the difference in slope of the response curves.

Examination of the protocols for negative control animals again points, albeit only slightly, to the superiority, that is, greater success in producing complete vitamin A deficiency, of the routine diet over the modified. (As negative control animals are included all animals that either received no supplement at all during the experimental period—"running out" time plus 35 days—or died within 7 days after receiving the first dose: nearly all the animals were in the former category. Such animals from the few litters not used in calculating the assay values have been included.) The protocols are summarized in Table 5. Animals of

Table 5. *Effect of diets on negative controls*

Sex	Diet	Mean no. of days* to death	Maximum gain* in weight	Mean gain* in weight at death	Incidence of xerophthalmia %
♂♂	Routine	38 (15)†	53 (13)	40 (13)	67 (12)‡
♂♂	Modified	48 (12)†	57 (12)	46 (12)	46 (13)
♀	Routine	43 (11)	51 (11)	37 (11)	73 (11)
♀	Modified	45 (18)	49 (18)	37 (18)	67 (18)

No. of animals in brackets.

* From weaning, at 20–25 days old.
† For the difference of these two means, $P = 0{\cdot}006$.
‡ 83%, including two animals with eye abscesses.

either sex die slightly earlier and show a slightly higher incidence of xerophthalmia on the routine diet than on the modified, though most of the differences appear to have a very low degree of statistical significance. The value of P, however, for the difference in mean ages at death of the two groups of male animals is 0·006, indicating a highly significant difference.

Some, if not all, of the results of this experiment that have been discussed might be attributed to the presence of traces of vitamin A in the coconut meal, but such an occurrence can hardly have any bearing on sex difference in response to doses of vitamin A. Speculation as to the relation of these differences in "slope" or discrimination, and still more of the differences in mean response, to the metabolism of carotene and vitamin A does not seem to us likely to serve a very useful purpose. We have been concerned rather with a study of methods of assay than of physiological processes. The results are presented in the hope that they may assist others who are involved in the difficult task of attempting to carry out quantitative measurement with biological material showing as low a variability as can be brought about by nature (inbreeding) and nurture (standardization of conditions before and during the experimental period).

It would seem pertinent in this connexion to refer here to certain other results obtained as a result of a large-scale experiment carried out by us some 3 years ago. These results serve to throw into relief a fact that is generally recognized, yet appears frequently to be overlooked by investigators making well-meaning efforts to increase the accuracy of biological tests to an extent rendered impossible by the nature of the experimental material.

In our earlier paper [Bacharach, 1933] we described broadly the genetic and environmental history of our experimental animals; we venture to claim that the

intensity of our drive towards uniformity could hardly be increased. Yet examination of the variability of our ordinary stock on a full diet, described in our earlier paper, shows how large a part of their variability under experimental conditions in assay work must be regarded as unavoidable. This is a matter that, like so many others of importance to bio-assayists, has already received the critical attention of Coward [1932]. She found the standard deviation of the weight-increase of vitamin A-deficient animals receiving test doses to be the same as that of the weight-increase of rats growing normally from the same average initial weight and for the same period. It seemed a matter of some interest, then, to examine the basic variability of normal animals.

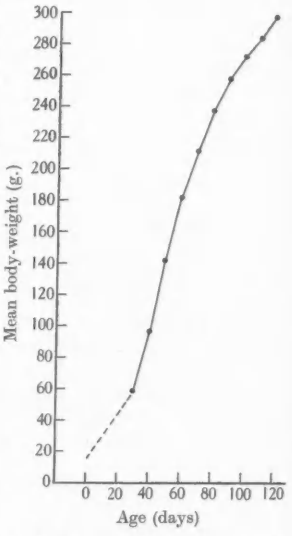


Fig. 2. Body-weight. Mean values for 105 ♂ receiving stock diet *ad lib.*

105 young males were taken from their mothers at weaning, in groups of 3 per litter. They were maintained until they were 120 days old on the same stock diet as that received by their mothers. They were weighed twice weekly and their weights at 10-day intervals estimated by interpolation. On plotting the mean of these weights a smooth average growth curve was obtained; this may be taken as representative of our male stock and its environment at the time of the experiment, and is shown in Fig. 2. By the end of the experiment the following figures were obtained:

No. of animals (males)	105
Range of weights (g.)	205–378
Mean weight $\overline{W}$	297
σ_W	30·3
$\epsilon_{\overline{W}}$	2·96
Coefficient of variation	10·2
$\overline{W} \pm 2/3 \times \sigma_W$	277; 317
$\overline{W} \pm \sigma_W$	267; 327
$\overline{W} \pm 2 \times \sigma_W$	236; 358

Thus, over half the animals will differ by more than 20 g. from the mean; one third will differ by more than 30 g. from the mean. Of every 22 animals one will differ by 60 g. from the mean.

Examination of the individual protocols failed to reveal any appearance of correlation between size of litter from which the triad had been taken and initial or final weight of the animals, or between these weights and the age (in days) at weaning, that is, removal from the mother. (Young rats begin to consume food for themselves several days before the usual "weaning" time of 20—23 days.)

It did, however, appear that there was a marked correlation between the initial and final weights of an animal. The animals were accordingly divided into two series, which had differed slightly in pre-experimental history, because the differences between the mean weights at 30 days old were significant ($P = 0{\cdot}001$), although those at 120 days were not ($P = 0{\cdot}45$). It was not legitimate, therefore, to test the correlation between initial and final weights on all the animals treated as one series, and the correlation coefficients were accordingly calculated for the

two series separately. The figures for initial and final body-weights are shown in Table 6. It will be noted that the animals in Series I remain consistently ahead of those in Series II, although the difference has ceased to be significant at 120 days, because of the intervening great increase in weights. These figures—and others in our possession—point clearly to the lasting effect of initial differences in weight; a poorer beginning confers a permanent handicap.

Table 6

		Body-weight (g.)				
		30 days		120 days		
Series	No. of animals	Mean	σ_W	Mean	σ_W	*P*- for correlation of initial and final wt.
I	51	61·5	9·43	299·1	35·37	$\ll 0{\cdot}001$ ($r = 0{\cdot}7914$)
II	54	55·8	7·33	294·9	24·49	0·005 ($r = 0{\cdot}4096$)
P- for difference between means		0·001		0·45		

Series	No. of animals	Body-weight (g.) at 30 days old		Increase in body-weight between 30 and 120 days		*P*- for correlation of initial wt. and wt. increase
		Mean	σ_W	Mean	σ_{dW}	
I	51	61·5	9·43	238·7	28·00	0·005 ($r = 0{\cdot}4100$)
II	54	55·8	7·33	238·9	22·31	No correlation ($r = 0{\cdot}1074$)
P- for difference between means		0·001		No significant difference		

In tests involving the use of weight increases, a more important consideration is the relation, if any, between the initial weight of an animal and its subsequent increase in weight. Inspection of the individual figures suggested that a correlation of this kind would be found in animals in Series I; calculation of the correlation coefficient showed this to be so. On the other hand, there was no correlation at all between initial weight and increase in weight of the animals in Series II. The animals in Series I were taken from our general breeding stock, and we have long regarded animals of this type as unsuitable for vitamin A assay, owing to their reserves of vitamin A being too varied and rather too high. The animals in Series II had, along with their mothers, undergone a pre-experimental period of vitamin A deficiency from about the 10th day after birth; further, the mothers themselves had, before maturity, been submitted to a vitamin A-deficient diet for 2–3 weeks. Only animals raised in this manner are used by us for work on vitamin A: the procedure is partly based on a recommendation of Nelson [1928], to which reference was made in the earlier papers of this series.

It will be observed also that the standard deviation of the values for the males in Series II is less than that of the values in Series I; the difference has a significance level of only 20% and can therefore be disregarded. Analysis of the results obtained with two similar groups of females (51 in Series I and 48 in Series II) also showed a difference in standard deviation. Here the animals in Series I varied less than those in Series II and the difference has a 1% level of significance. It is difficult to understand how a procedure—pre-experimental restriction of ingested vitamin A—which has had no effect on the variability of the males can have increased the variability of females.

On the other hand, the correlations observed with males were exactly paralleled in the females. In Series I initial weight was strongly correlated with increase in weight ($r = 0{\cdot}343$; $P = 0{\cdot}015$). In Series II this correlation disappeared; indeed it became slightly, though not significantly, negative ($r = -0{\cdot}073$). There seems little doubt, therefore, that the pre-experimental treatment of both males and females has had the same effect: cutting down vitamin A reserves to rock-bottom has eliminated—at any rate for animals receiving a full stock diet—the normally marked effect of original weight on rate of growth (weight-increase) during the 5th to 16th weeks of life. There seems no reason to doubt that the effect in question would also be eliminated in animals receiving a vitamin A-free diet.

The practical conclusion would seem to be that, if animals are taken direct from unprepared stock for tests involving increases in weight, then they should be arranged in sets of identical initial weight. This conclusion is, in itself, a justification for using litter-mate (isogenic) sets, because variations in weight of animals of the same sex and litter can be seen from a large number of records, our own and others, to be much less than the variations between the mean weights of groups of isogenic animals. In vitamin A assays, however, this special arrangement of the animals may be unnecessary, provided that a suitable depletion period has secured that the weight increases of the animals are independent of their initial weights.

Although the figures given in the later part of this paper are derived from a relatively large sample of normal animals, they indicate to how great an extent the variance of a smaller group of animals, receiving an experimental—that is, a deficient—diet, is due to inherent variability that is entirely independent of the experimental conditions imposed during a vitamin assay.

REFERENCES

Bacharach (1933). *Biochem. J.* **27**, 5.
—— & Lester Smith (1933). *Biochem. J.* **27**, 17.
Coward (1932). *Biochem. J.* **26**, 691.
—— (1933). *Biochem. J.* **27**, 445.
—— Dyer, Morton & Gaddum (1931). *Biochem. J.* **25**, 1102.
Irwin (1937). *Suppl. J. Roy. Stat. Soc.* **4**, 1.
de Loureiro (1931). *Arquito Patolog.* **3**, 72.
Maitra & Moore (1939). *Biochem. J.* **33**, 1648.
Mead, Underhill & Coward (1939). *Biochem. J.* **33**, 589.
Nelson (1928). *Science*, **68**, 212.
Richards & Simpson (1934). *Biochem. J.* **28**, 1274.
Tainsh & Wilkinson (1939). *Chem. Ind. Rev.* **58**, 1051.

67. ADRENALINE HYPERGLYCAEMIA

By MARGARET LEE and DEREK RICHTER

From the Central Pathological Laboratory, West Park Hospital, Epsom

(*Received 21 February 1940*)

Adrenaline hyperglycaemia is mainly due to an increased breakdown of glycogen in the liver, but how adrenaline produces this effect is not known. Until recently it was believed that adrenaline activates the liver amylase or brings the amylase and glycogen together so that they can interact [Willstätter & Rohdewald, 1936; Lesser, 1921], but it is now known that the liver contains a phosphorylating system which is also able to break down glycogen to glucose, and it has been suggested that adrenaline may act by accelerating the phosphorylysis of glycogen [Cori & Cori, 1938; Cori *et al.* 1939; Ostern *et al.* 1939].

In a previous investigation evidence was obtained which made it appear unlikely that the glycogenolysis in adrenaline hyperglycaemia is due to the liver amylase [Lee & Richter, 1940]. In the present study an attempt was made to obtain further information as to (*a*) whether the activity of the liver phosphorylase is sufficient to account for the rate of glycogenolysis in adrenaline hyperglycaemia and (*b*) how the phosphorylysis of glycogen is influenced by factors which might be expected to play a part in regulating the rate of glycogen breakdown in the liver under physiological conditions.

Methods

The activity of the liver phosphorylase was determined by incubating liver brei with an excess of glycogen in the presence of NaF and measuring the uptake of inorganic phosphate due to the formation of hexosemonophosphate. The glycogen was prepared and the glycogen and phosphate were estimated by the methods described by Lee & Richter [1940]. In order to ensure the activity of the phosphorylating system adenylic acid, $MgCl_2$ and, in some experiments, HCN were added. In preparing the liver brei, the liver, which was excised under nembutal anaesthesia except where otherwise stated, was cooled on ice, ground thoroughly with sand in a cooled mortar and mixed with an equal volume of water at 0°. Rabbit livers were generally used. In experiments carried out under conditions "A" a total volume of 5 ml. contained 2 ml. liver brei, 0·1 ml. 0·1% adenylic acid, 0·5 ml. *M*/9 Sörensen phosphate buffer *p*H 7·0, 0·1 ml. $MgCl_2$ solution (1·0 mg. Mg/ml.), 0·5 ml. *M*/2 NaF and sufficient glycogen solution to bring the glycogen concentration, with that already present in the liver, to 1–2%. Tubes containing the mixture were incubated in a bath at 37° and samples of 0·5 ml. were taken at the beginning and at various time intervals for phosphate and glycogen estimations. Experiments carried out under conditions "B" contained 0·5 ml. *M*/2 HCN, prepared by neutralizing KCN with HCl, in addition to the substances present under conditions "A".

The figures giving the phosphorylysis and amylolysis (glycogen breakdown by amylase) are calculated in terms of mg. glycogen broken down per g. fresh liver per hr. The phosphorylysis figures were calculated directly from the uptake of inorganic phosphate due to hexosemonophosphate formation. The amylolysis

figures represent the total glycogen breakdown as given by the glycogen estimations *minus* the glycogen breakdown due to phosphorylysis.

Blood sugar estimations were done by the Hagedorn & Jensen [1923] method. The additions were neutralized when necessary with NaOH or HCl; the figures giving the amounts added are per g. fresh tissue. Phosphate uptakes are given as mg. P per g. fresh tissue.

Rate of glycogenolysis in vivo

Assuming that the rise in blood sugar in adrenaline hyperglycaemia is mainly due to the breakdown of liver glycogen, it is possible to estimate the rate of glycogenolysis in the liver that would be necessary to account for an observed blood sugar rise.

Rabbits that had been well fed with carbohydrate were deprived of food for 18 hr. and blood sugar estimations were done before and after subcutaneous injection of adrenaline (0·04–0·45 mg./kg.). The blood samples were taken from the marginal ear vein and the glucose estimations done in duplicate.

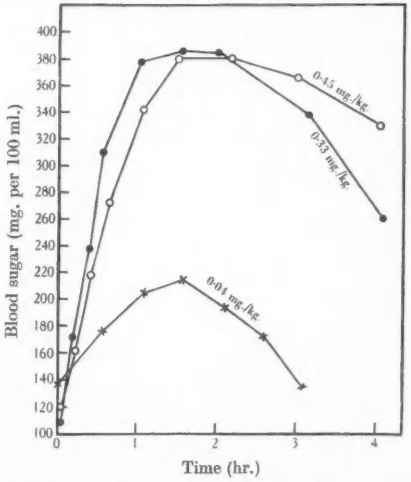


Fig. 1. Adrenaline hyperglycaemia in the rabbit. (Adrenaline doses are shown on curves.)

The steepest part of each curve gave the maximal rate of blood sugar rise. The rate of rise in the blood sugar is known to increase with increasing adrenaline doses until it reaches a maximal rate which is not further increased by giving larger amounts of adrenaline [Griffith *et al.* 1939]; this may be due to the rate of rise being limited by the maximum possible rate of glycogenolysis in the liver. Taking the liver weight as 4·5 % of the body weight and assuming that under these conditions the blood sugar is in equilibrium with 20 % of the body weight [cf. Evans *et al.* 1931], the total amount of sugar (x) passed into the blood in a given time (t) and hence the rate of glycogenolysis (y) in the liver could be estimated $\left(y=\frac{x}{100}\times\frac{20}{4{\cdot}5}\times\frac{60}{t}\right)$. The amount of sugar passing into the urine under these conditions was taken as being negligible.

Exp. no.	Adrenaline mg./kg.	Time interval min.	Blood sugar rise mg./100 ml.	Glycogenolysis rate in liver mg./g./hr.
1	0·04	12	124 → 180 = 45	10
2	0·33	10	239 → 311 = 72	19
3	0·45	11	161 → 218 = 57	14
4	0·33	14	249 → 375 = 126	24

In this way it was estimated that, in the intense hyperglycaemia induced by adrenaline, the system responsible for glycogenolysis in the liver must be capable of breaking down 15–25 mg. glycogen per g. liver per hr. These figures can be taken as giving only a very rough estimate of the rate of glycogenolysis that may be expected to occur *in vivo*, but they are of the same order as the figures obtained by Evans *et al.* [1931] who carried out direct glycogen estimations on liver samples removed during adrenaline hyperglycaemia in the decapitated cat.

Activity of liver phosphorylase

Lability of phosphorylase. Cori & Cori [1938] found that the liver phosphorylase was easily inactivated during dialysis; it was frequently confirmed in the present work that the activity diminished in a few hours at 0° and generally disappeared completely if the liver or brei was left overnight in the ice-chest. In this respect the phosphorylase differed markedly from the liver amylase, which remained active on keeping for several days:

Exp. no	Age of brei	Incubation hr.	Amylolysis mg./g./hr.	Phosphorylysis mg./g./hr.
1	4 days at 4°	2	13·0	0
2	1 day at 4°	3	2·14	0
3	1 hr. at 37°	1½	6·7	0

The rate of inactivation of the phosphorylase was much greater at 37° and little or no phosphorylation could be detected in liver breis that had been kept at 37° for 1–2 hr. The observed inactivation was not due to the accumulation of glucose or other reaction products since attempts to reactivate the enzyme by dialysis were unsuccessful.

These experiments were carried out with rabbit liver under conditions "B", and under the same conditions the fresh liver showed an average phosphorylysis rate of 1–3 mg. glycogen per g. liver per hr. during an incubation of 1–3 hr. The times of incubation were similar to those used by other investigators who have studied glycogenolysis in the liver, but it was evident that experiments of such long duration could give little indication of the true activity of a system so labile as the liver phosphorylase.

When phosphate estimations were done after much shorter incubation times it was seen that most of the phosphate uptake occurred in the first few minutes of the incubation (Fig. 2). After about 1 hr. there was even a diminution in the phosphate uptake, which indicated that the decomposition of phosphate esters was not negligible under these conditions in experiments of long duration. In this respect the liver system showed a difference from the muscle system, a difference which may be attributed to the very active phosphatase present in liver.

In a number of experiments in which the activity of the liver phosphorylase was estimated in different animals by incubating the liver brei under similar conditions for periods of 10–20 min., much higher phosphorylysis rates were

obtained (Table 1). Since the present investigation was completed Ostern *et al.* [1939] have also reported experiments showing high phosphorylation rates in liver brei.

Table 1. *Rate of phosphorylysis in liver brei*

Exp. no.	Animal	Conditions	Incubation time min.	Initial phosphate mg. P/g.	P uptake mg. P/g.	Phosphorylysis mg./g./hr.
1	Rabbit	B	20	1·94	0·88	13·7
2	,,	B	15	1·23	0·87	7·5
3	,,	A	10	1·79	0·43	13·4
3	,,	B	10	1·87	0·53	16·6
4	Guinea-pig	A	5	2·10	0·20	13·2
5	,,	A	5	1·06	0·26	16·4
6	,,	B	15	1·87	0·81	12·6
7	,,	A	5	3·23	0·32	20·0

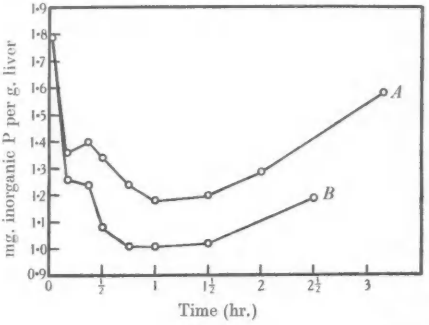


Fig. 2.

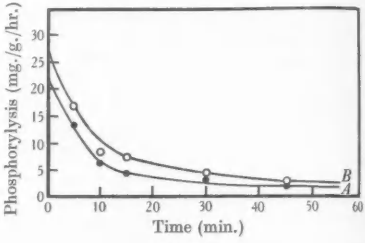


Fig. 3.

Fig. 2. Phosphate uptake at different times. (Conditions "A" and "B".)

Fig. 3. Rate of phosphorylysis observed with different incubation times. (Conditions "A" and "B".)

The falling off in the phosphorylation rate with longer incubation times might be attributed to the drop in phosphate concentration, which would be expected to reduce the rate; that the falling off was in fact mainly due to the inactivation of the enzyme was shown by comparing the activities of samples to which the same amount of phosphate was added after keeping at 37° and at lower temperatures.

Exp. no.	Treatment of brei	Incubation time min.	Phosphorylysis mg./g./hr.
1	15 min. at 10°	15	6·7
	15 min. at 37°	15	2·7
2	15 min. at 0°	15	4·5
	15 min. at 37°	15	1·2
	30 min. at 37°	15	0

Curves obtained by plotting the observed phosphorylysis rate against the incubation time (Fig. 3) indicated that the phosphorylase activities observed with shorter incubation periods were still below the true initial activities in the brei: by extrapolation to zero time higher phosphorylysis rates of the order of 20–30 mg./g./hr. were obtained. It was therefore evident that the activity of the liver phosphorylase was sufficient to account for the rates of glycogenolysis observed in adrenaline hyperglycaemia *in vivo*.

Factors affecting the rate of phosphorylysis

Glucose. In considering the mechanism by which the activity of the liver phosphorylase is controlled *in vivo*, the observation of Gill & Lehmann [1939] that the muscle phosphorylase is inhibited by glucose suggested that a similar mechanism might operate *in vivo* in the liver, and the phosphorylysis of glycogen in the liver might be controlled by the local glucose concentration. The inhibition by glucose of the phosphorylysis in muscle extract was readily confirmed:

Exp. no.	Age of muscle extract days	Incubation time hr.	Initial phosphate mg. P/g.	Phosphate uptake mg. P/g.	Inhibition by 6% glucose %
1	3	3	2·10	0·74	100
2	2	3	1·95	0·44	73
3	6	3	2·06	0·31	100
4	1	3	1·96	0·46	100

The muscle extract was prepared by the method of Meyerhof [1926]. Each tube contained 2·5 ml. muscle extract, 0·3 ml. *M*/9 Sörensen phosphate buffer *p*H 7·0, 1 ml. 5% glycogen, 0·1 ml. 0·1% adenylic acid, 0·1 ml. $MgCl_2$ solution containing 1 mg. Mg/ml., 0·5 ml. *M*/2 HCN, 0·1 ml. 5% NaCl and 1 ml. water or glucose solution in a total volume of 5·6 ml. The initial phosphate and phosphate uptake figures are expressed as mg. P in each tube.

The phosphorylysis of glycogen in liver brei was also inhibited by glucose, but the inhibition was generally less than with the muscle extract and showed considerable variation from one animal to another (Table 2).

Table 2. *Inhibition of phosphorylysis in liver by 6% glucose*

Exp. no.	Animal	Conditions	Incubation time hr.	Initial phosphate mg. P/g.	Phosphate uptake mg. P/g.	Inhibition by 6% glucose (%)
1	Rabbit	A	1	0·96	0·39	41
2	,,	B	1	2·23	0·28	100
3	,,	B	1	1·47	0·56	82
4	,,	B	2¼	1·32	0·49	43
5	,,	A	½	1·23	0·35	49
6	,,	B	½	1·16	0·44	59
7	Guinea-pig	B	2	1·24	0·63	46

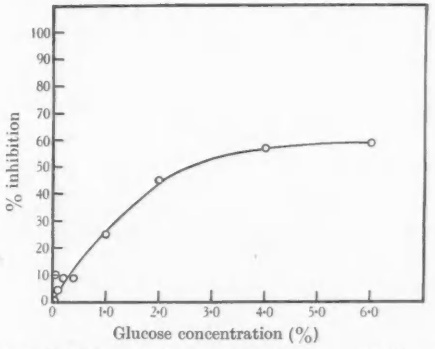


Fig. 4. Inhibition of phosphorylysis by glucose at different concentrations. (Conditions "B".)

At lower glucose concentrations, such as might be expected to obtain under physiological conditions, the inhibition was relatively small (Fig. 4). It therefore

appeared unlikely that a direct inhibition of the phosphorylase by glucose could play a significant part in controlling the rate of glycogenolysis in the liver.

Oxidizing and reducing agents. Lipmann [1933] showed that the phosphorylysis of glycogen by muscle extract is inhibited reversibly by oxidizing agents and suggested that the inhibition of glycolysis by oxygen (Pasteur effect) might be explained in this way. Gill & Lehmann [1939] have extended Lipmann's observations to other oxidizing and reducing agents and reported a marked increase in the phosphorylysis rate in the presence of hydrogen cyanide.

The observations of Gill & Lehmann were readily confirmed with muscle extracts under the conditions of the present experiments:

Initial phosphate mg. P	Phosphate uptake alone mg. P	Addition	Phosphate uptake with addition mg. P	Inhibition %	Activation %
1·64	0·20	Sat. quinhydrone	0·00	100	—
1·77	0·31	*M*/20 HCN	0·80	—	160
2·07	0·25	*M*/20 HCN	0·46	—	84
1·67	0·14	20 mg. thiolacetic acid	0·43	—	207
1·67	0·14	5 mg. GSH	0·16	—	14
1·67	0·14	50 mg. ascorbic acid	0·30	—	114

Each tube contained 2·0 ml. muscle extract prepared by the method of Meyerhof [1926], 1·0 ml. 5% glycogen, 0·1 ml. $MgCl_2$ (containing 1 mg. Mg/ml.), 0·1 ml. 0·1% adenylic acid, 0·1 ml. 5% NaCl, 0·3 ml. *M*/9 Sörensen phosphate buffer *p*H 7·0, 0·5 ml. *M*/2 NaF, 0·1 ml. *M*/1·5 $NaHCO_3$ and special addition or water to bring the total volume to 5·0 ml. The phosphate figures give mg. P per tube. The incubation time was 1–2 hr.

Owing to the very active reducing systems present in liver brei it was necessary to add a relatively large amount of oxidizing agent. A strong inhibition was obtained when autoxidizable dyes were used and the tubes were rapidly shaken in the water bath:

Initial phosphate mg. P/g.	Phosphate uptake alone mg. P/g.	Addition	Phosphate uptake with addition mg. P/g.	Inhibition %
1·67	0·17	10 mg. 2:6-dichlorophenolindophenol (not shaken)	0·21	0
1·16	0·25	20 mg. 2:6-dichlorophenolindophenol (shaken)	0·00	100
1·32	0·24	20 mg. 2:6-dichlorophenolindophenol (shaken)	0·04	83
1·79	0·45	2 mg. methylene blue (shaken)	0·22	51
1·79	0·45	2 mg. K-indigodisulphonate (shaken)	0·21	53
1·79	0·45	2 mg. α-naphthol-2-Na-sulphonate indophenol (shaken)	0·45	0

Reducing agents such as glutathione, thiolacetic acid and ascorbic acid had little effect on the rate of phosphorylysis in liver brei, as might be expected in view of the large amounts of similar reducing agents already present, but a strong acceleration by HCN was observed:

Initial phosphate mg. P/g.	Phosphate uptake alone mg. P/g.	Addition	Phosphate uptake with addition mg. P/g.	Change %
1·12	0·34	5 mg. GSH	0·39	+ 15
1·12	0·34	20 mg. thiolacetic acid	0·26	− 23
1·12	0·34	50 mg. ascorbic acid	0·32	− 6
1·12	0·34	*M*/20 HCN	0·70	+106
1·67	0·17	*M*/20 HCN	0·56	+229

Release of inhibition by HCN

The effect of HCN in increasing the rate of phosphorylysis was very striking. It indicated that the rate of phosphorylysis was not maximal in fresh liver brei, but that the system was partially inhibited. The effect of HCN in releasing that inhibition was studied further in the hope of obtaining information as to the factors which normally inhibit the phosphorylysis of glycogen under physiological conditions. The HCN effect was confirmed in a great many experiments under different conditions, using perfused and unperfused livers, different incubation times and the livers from different species. The magnitude of the effect differed from one animal to another, but there was nearly always a marked increase in the phosphorylysis rate (Table 3).

Table 3. *Effect of HCN on phosphorylysis in liver brei*

Exp. no.	Animal	Perfusion	Incubation time hr.	Initial phosphate mg. P/g.	Phosphate uptake mg. P/g. Alone	Phosphate uptake mg. P/g. With HCN	Increase with HCN %
1	Rabbit	—	1½	1·03	0·48	0·70	46
2	,,	—	1	1·84	0·69	0·85	23
3	,,	Perfused	1¾	0·97	0·14	0·32	128
4	,,	,,	2	1·16	0·25	0·41	64
5	,,	,,	½	1·61	0·26	0·72	177
6	,,	,,	½	1·12	0·32	0·44	37
7	,,	,,	½	1·79	0·45	0·70	56
8	Guinea-pig	—	2	1·24	0·25	0·63	152
9	,,	—	¼	1·53	0·48	0·81	69

Perfusion with saline as described by Lee & Richter [1940].

The rate of glycogenolysis in the tissues is known to be increased by anoxaemia, and it was thought that HCN might be acting by inhibiting the transfer of O_2 by the cytochrome-cytochrome oxidase system. Against this it was found that added cytochrome *c* produced no effect, and no acceleration was observed with 0·1% NaN_3 or *M*/1000 HCN, which are sufficient to produce a very considerable inhibition of the cytochrome system. Experiments in which the air was evacuated from the tubes or replaced by coal gas also gave no significant change in the phosphorylysis rate (Table 4).

Table 4. *Relation of the cytochrome system to phosphorylysis*

Exp. no.	Incubation time hr.	Initial phosphate mg. P/g.	Phosphate uptake alone mg. P/g.	Addition	Phosphate uptake with addition mg. P/g.	Change %
1	½	1·79	0·45	1·5 mg. cytochrome *c*	0·46	+ 2
2	½	1·97	0·15	3 mg. cytochrome *c*	0·13	− 13
3	½	1·61	0·26	Evacuated	0·28	+ 8
4	½	1·97	0·15	Coal gas	0·14	− 7
5	½	1·79	0·45	1% NaN_3	0·38	− 15
6	½	1·32	0·24	,,	0·17	− 29

Conditions "A"; rabbit liver used.

Phosphorylysis is known to be strongly inhibited by traces of Cu and other heavy metals, but it was unlikely that the HCN was acting by removing Cu,

since the addition of sodium diethyldithiocarbamate, which specifically combines with Cu, produced no effect:

Initial phosphate mg. P/g.	Phosphate uptake alone mg. P/g.	Addition	Phosphate uptake with addition mg. P/g.	Change %
1·53	0·48	Na diethyldithio-carbamate (5 mg.)	0·42	−12

Guinea-pig liver used; incubation period ¼ hr.

A further possibility to be considered was that the HCN was acting by removing an aldehyde or ketone which was serving as an inhibitor. In support of this view, a number of aldehydes were found to inhibit the phosphorylysis; but semicarbazide or hydroxylamine failed to show any acceleration such as was produced by HCN.

Exp. no.	Phosphate uptake alone mg. P/g.	Addition	Phosphate uptake with addition mg. P/g.	Change %
1	0·48	1% acetaldehyde	0·14	−71
2	0·48	1% *n*-butaldehyde	0·12	−75
3	0·48	1% octaldehyde	0·16	−67
4	0·48	2% Na pyruvate	0·39	−19
5	0·48	*M*/2·5 semicarbazide	0·46	− 4
6	0·48	*M*/2·5 hydroxylamine	0·41	−15
7	0·48	*M*/20 HCN	0·81	+69

Conditions "A"; guinea-pig liver. Incubation time 15 min. Initial phosphate 1·53 mg. P/g. liver.

Gill & Lehmann [1939] concluded that the phosphorylase is inactive under oxidizing conditions and that HCN and the other reducing agents activate it by reducing —SS— groups in the enzyme to —SH. It would appear doubtful, however, whether the phosphorylase may be always inactive under oxidizing conditions, since it is believed to be concerned in the resynthesis of glycogen from glucose, which occurs only in the presence of O_2. The evidence suggests rather that HCN may not act directly on the phosphorylase, but indirectly by removing an inhibitory reaction product.

Effect of adrenaline on phosphorylysis

In several experiments in which varying amounts of adrenaline were added to the liver brei it was found, in agreement with Ostern *et al.* [1939], that no effect on the rate of phosphorylysis could be observed.

If adrenaline works *in vivo* by activating the liver phosphorylase it appeared that it might be possible to show an increased phosphorylase activity in samples of liver rapidly taken out during adrenaline hyperglycaemia. In order to test this, two glass pestles and mortars containing sand were set up in a covered water bath at 37°. A guinea-pig or rabbit was anaesthetized with nembutal, the abdomen opened and a lobe of liver tied off with cotton and removed. The lobe was rapidly weighed and ground with sand in a mortar at 37°. The weighing occupied less than 1 min. After grinding for 1 min., a solution containing phosphate, glycogen and NaF kept at 37° was added, and the phosphorylase activity was tested by measuring the rate of phosphate uptake in the usual way.

After the removal of the first lobe of liver the abdomen was closed, the animal was kept warm by surrounding with cotton wool and placing near an electric heater and adrenaline was injected subcutaneously. 5 min. later a second lobe

Table 5. *Phosphorylase activity of liver removed during adrenaline hyperglycaemia*

Guinea-pig 1			Guinea-pig 2			Rabbit 1		
Incubation min.	Lobe 1 (1·9 g.) normal	Lobe 2 (2·1 g.) 5½ min. after adrenaline	Incubation min.	Lobe 1 (3·5 g.) normal	Lobe 2 (2·8 g.) normal	Incubation min.	Lobe 1 (10·0 g.) normal	Lobe 2 (10·2 g.) 17 min. after adrenaline
5	13·2	11·25	5	16·4	14·0	8	7·3	—
10	13·2	9·35	10	11·0	11·4	16	4·6	4·0
15	10·0	8·3	15	9·6	8·3	24	3·7	3·3

Phosphorylase activities expressed as mg. glycogen broken down per g. fresh liver per hr. Guinea-pig 1 received 0·3 mg. adrenaline per kg. Guinea-pig liver lobes were ground with 2·5 ml. 5% glycogen, 2·0 ml. *M*/2 NaF and 0·25 ml. *M*/1·5 Sörensen phosphate buffer *p*H 7·0. Rabbit 1 received 0·4 mg. adrenaline per kg. The rabbit liver lobes were ground with double these quantities of glycogen, etc.

of liver was removed and tested in the same way as the first. A slight diminution in phosphorylase activity was observed in the successive lobes and there was the usual decrease in activity during the incubation. The experiments showed that the phosphorylase in the normal liver tested under these conditions was already very active, and no further activation by adrenaline was observed.

Effect of adrenaline on inorganic phosphate and adenosine triphosphate in liver

Apart from a direct activation of the phosphorylase, adrenaline might increase the rate of phosphorylysis in the liver (*a*) by increasing the concentration of inorganic phosphate, (*b*) by catalysing the decomposition of adenosine triphosphate and so liberating phosphate and adenylic acid which activates the phosphorylase or (*c*) by hastening the removal of glucose-1-phosphate or other inhibitory reaction products.

Orientating experiments failed to show any very striking difference from the normal in the inorganic phosphate or adenosine triphosphate concentration in livers taken out after administering adrenaline. In these experiments the liver samples were removed with the greatest possible speed and ground with trichloroacetic acid. In the guinea-pig experiments, the carotid arteries were cut immediately after giving the animal a blow on the head; the amount of

Table 6. *Phosphate and adenosine triphosphate concentrations during adrenaline hyperglycaemia*

Exp. no.	Animal	Adrenaline dose (mg./kg. subcut.)	Duration of adrenaline action min.	Inorganic phosphate + phosphagen mg. P/g.	A.T.P. mg. P/g.
1	Guinea-pig	—	—	0·262	0·148
2	,,	—	—	0·318	0·104
3	,,	0·2	20	0·269	0·153
4	,,	1·0	30	0·299	0·104
5	Rabbit	—	—	0·293	0·054
5	,,	0·4	17	0·289	0·049

Inorganic phosphate was estimated by the method of Denigès [1921] which includes creatine phosphate; but the amount of creatine phosphate in the liver is relatively small [Takahisa, 1936; Wajzer & Lippmann, 1938]. Exp. 5 was done by removing lobes of liver before and after adrenaline injection. A.T.P. was estimated by the method of Lohmann [1928].

adrenaline reaching the liver from the adrenals before the carotids were cut was probably negligible. The amount of inorganic phosphate found in the trichloroacetic acid filtrate should be sufficient to produce a rapid rate of phosphorylysis if present in the free form *in vivo*.

Discussion

Liver amylase and phosphorylase. It has been estimated that the system responsible for the glycogenolysis in the liver during adrenaline hyperglycaemia must be capable of breaking down 15–25 mg. glycogen per g. liver per hr. The activity of the amylase found in the liver is frequently too low to account for so rapid a rate of glycogenolysis, and in a previous paper other reasons have been given for believing that the amylase is not concerned to any significant extent in glycogenolysis in the liver [Lee & Richter, 1940].

The liver phosphorylase is very labile and therefore may appear to be inactive in experiments of long duration, but the activity of the phosphorylase in the fresh liver tissue is very great, being similar to that in muscle and amply sufficient to account for the rate of glycogenolysis observed during adrenaline hyperglycaemia *in vivo*.

Normal blood sugar control. Under normal conditions there must be a balance between glycogen formation from glucose and glycogenolysis in the liver. Assuming that the liver amylase is not involved, the fasting blood sugar level must be maintained by a rate of phosphorylysis which is normally relatively slow: under normal conditions the phosphorylysis of glycogen must be largely inhibited *in vivo*.

It has been shown that the blood sugar level is controlled to a considerable extent by the liver alone, apart from any nervous or hormonal mechanism, since the blood sugar level is maintained approximately constant after denervating the liver, after sympathectomy or after removal of the pituitary gland [Etcheverry, 1937; Soskin *et al.* 1939; Brouha *et al.* 1939]. This means that the rate of phosphorylysis in the liver must depend on the blood sugar level, and phosphorylysis must be increasingly inhibited as the blood glucose rises.

The secondary rise in the blood sugar produced in response to the injection of insulin and many other stimuli is believed to be mediated by adrenaline. Adrenaline, insulin and the pituitary hormones adjust the position of the balance between glycogen synthesis and glycogen breakdown, but they do so by acting on a regulatory mechanism in the liver cells, a mechanism which would appear to depend on the inhibition of phosphorylysis by glucose.

Under the influence of anoxaemia the normal inhibition of phosphorylysis is removed: a rapid glycogen breakdown is observed and the blood sugar may rise to a high value without phosphorylysis being inhibited. Phosphorylysis is therefore inhibited by glucose only in the presence of oxygen. A direct inhibition of the phosphorylase by glucose is unlikely since phosphorylysis in the liver brei was inhibited only 50 % by 6 % glucose, and at glucose concentrations which might be possible under physiological conditions the inhibition was negligible. The inhibitor which operates *in vivo* is apparently a substance which is produced from glucose in the presence of oxygen; this might be glucose-1-phosphate, which is a powerful inhibitor of phosphorylysis even at low concentrations.

$$\text{Glycogen} + H_3PO_4 \rightleftharpoons \text{glucose-1-phosphate} \underset{\text{oxygen}}{\overset{\text{anoxaemia}}{\rightleftharpoons}} \text{glucose} + H_3PO_4.$$

Action of adrenaline. Hegnauer & Cori [1934] have shown that adrenaline increases the rate of phosphorylysis in muscle, and it might therefore be expected to act in an analogous manner on the phosphorylating system in the liver. A direct activation of the liver phosphorylase by adrenaline is unlikely since the phosphorylase catalyses the synthesis as well as the breakdown of glycogen, and an activation of the phosphorylase would be as likely to accelerate either process. It is clear that to increase the rate of phosphorylysis adrenaline must alter the concentrations of the reactants by increasing the effective concentrations of phosphate and glycogen or decreasing the concentration of the primary reaction product, which is believed to be glucose-1-phosphate.

The inorganic phosphate concentration found in the liver was generally about 0·3 mg. P per g., which should be sufficient to account for a rapid rate of phosphorylysis, and no rise was observed after adrenaline. A change in the effective glycogen concentration might be produced, as Wajzer [1938] has suggested, by the conversion of desmo- into lyo-glycogen; but most of the glycogen in the liver is already in the lyo-form. It is therefore probable that adrenaline increases the rate of phosphorylysis by accelerating the removal of glucose-1-phosphate or another phosphate ester intermediary between glycogen and glucose-6-phosphate.

The effects of adrenaline on glycogenolysis may be compared with the effects of anoxaemia: in muscle both anoxaemia and adrenaline increase the conversion of glycogen into lactic acid, while in the liver they both cause an increased conversion of glycogen into glucose.

There is a marked reduction in the blood flow due to vasoconstriction when adrenaline is injected into the perfused cat or rabbit liver [Clark, 1928; Neubauer, 1912], and Masing [1912] observed that after adrenaline the blood passing through the perfused liver may be completely reduced. The hyperglycaemic action of adrenaline has been attributed to the anoxaemia produced through vasoconstriction in the liver, but while this effect may be significant in some animals, it cannot be the only way in which adrenaline acts since adrenaline produces hyperglycaemia without a corresponding vasoconstriction in the perfused frog liver [Fröhlich & Pollack, 1914] and it also acts on isolated muscle [Hegnauer & Cori, 1934] and on tissue slices [Cross & Holmes, 1937].

The rate of phosphorylysis is increased by anoxaemia in the intact liver or by reducing agents and HCN in the liver brei. The precise manner in which adrenaline acts is still not clear, but it has been shown that adrenaline is able to act as an oxidation carrier [Green & Richter, 1937] and this suggests that adrenaline might act in producing a similar effect to anoxaemia by coupling the phosphorylating system with the reducing systems of the cell.

Summary

1. During adrenaline hyperglycaemia in the rabbit the system responsible for glycogenolysis in the liver is able to break down 15–25 mg. glycogen per g. liver per hr.

2. The activity of the liver phosphorylase is sufficient to account for the rate of glycogenolysis occurring in adrenaline hyperglycaemia *in vivo*.

3. The phosphorylysis of glycogen is inhibited by glucose, but the inhibition is not sufficient to account for the inhibition which occurs *in vivo*.

4. The phosphorylysis of glycogen in liver brei is inhibited by oxidizing agents and strongly accelerated by $M/20$ HCN.

5. The accelerating effect of HCN is not due to an inhibition of the cytochrome system.

6. No evidence was obtained that adrenaline works by activating the phosphorylase, by increasing the concentration of inorganic phosphate or by liberating adenylic acid from adenosine triphosphate.

The authors thank Prof. S. Nevin for his encouragement and the Rockefeller Foundation for supporting this investigation.

REFERENCES

Brouha, Cannon & Dill (1939). *J. Physiol.* **95**, 431.
Clark (1928). *J. Physiol.* **66**, 274.
Cori & Cori (1938). *Proc. Soc. exp. Biol., N.Y.*, **39**, 337.
—— —— & Schmidt (1939). *J. biol. Chem.* **129**, 629.
Cross & Holmes (1937). *Brit. J. exp. Path.* **18**, 370.
Denigès (1921). *C.R. Soc. Biol., Paris*, **17**, 1974.
Etcheverry (1937). *Rev. Soc. argent. Biol.* **13**, 74.
Evans, Tsai & Young (1931). *J. Physiol.* **73**, 87.
Fröhlich & Pollack (1914). *Arch. exp. Path. Pharmak.* **77**, 265.
Gill & Lehmann (1939). *Biochem. J.* **33**, 1151.
Green & Richter (1937). *Biochem. J.* **31**, 596.
Griffith, Lockwood & Emery (1939). *Amer. J. Physiol.* **126**, 299.
Hagedorn & Jensen (1923). *Biochem. Z.* **135**, 48.
Hegnauer & Cori (1934). *J. biol. Chem.* **105**, 691.
Lee & Richter (1940). *Biochem. J.* **34**, 353.
Lesser (1921). *Biochem. Z.* **119**, 108.
Lippmann (1933). *Biochem. Z.* **265**, 133.
Lohmann (1928). *Biochem. Z.* **194**, 306.
Masing (1912). *Arch. exp. Path. Pharmak.* **69**, 431.
Meyerhof (1926). *Biochem. Z.* **178**, 395.
Neubauer (1912). *Biochem. Z.* **43**, 335.
Ostern, Herbert & Holmes (1939). *Biochem. J.* **33**, 1858.
Soskin, Levine & Taubenhaus (1939). *Proc. Soc. exp. Biol., N.Y.*, **42**, 689.
Takahisa (1936). *Mitt. med. Akad. Kioto*, **17**, 615.
Wajzer (1938). *Bull. Soc. Chim. biol., Paris*, **20**, 828.
—— & Lippmann (1938). *Bull. Soc. Chim. biol., Paris*, **20**, 342.
Willstätter & Rohdewald (1936). *Enzymologia*, **1**, 213.

68. GLYCOLYSIS IN RETINAL EXTRACTS

By MARGARET KERLY and MARGHERITA COTONIO BOURNE
From the Department of Biochemistry, University College, London

(*Received 29 February 1940*)

The relatively high glycolytic power of retinal cells *in vitro* has been recognized since the work of Warburg *et al.* [1924], but it is still not known whether this activity is of any significance in the metabolism of living retina, nor have the intermediary steps of this glycolysis been worked out.

As a preliminary to a series of studies on retinal metabolism we have prepared a cell-free extract of retina [Kerly & Bourne, 1939] which fortunately proved to be very active glycolytically, and we have endeavoured to compare its behaviour with that of cell-free extracts of other tissues described by other workers.

The chief points of interest which emerge from the present work are concerned with the mechanism by which glucose is converted into lactic acid and with the question of the phosphorylation of glucose during the glycolysis. It has been suggested that the *in vitro* conversion of glucose into lactic acid by the cells of retina [Bumm & Fehrenbach, 1931] and of other tissues proceeds by a mechanism which is entirely different from that involved in the conversion of glycogen into lactic acid in tissue extracts. The presence of phosphate is said to be essential for glycogenolysis but unnecessary for glucolysis (see Needham & Lehmann [1937] for a discussion of the literature). The evidence for this hypothesis is unsatisfactory, since it is derived from experiments on intact cells where considerations of permeability and of the composition of the medium inside the cell make it difficult to interpret the results. In those cases where cell-free extracts which will attack glucose have been prepared [Euler *et al.* 1936; Süllman, 1937; Meyerhof, 1938], the evidence does not support the view that there are two separate mechanisms, one for the breakdown of glucose and another for the breakdown of glycogen; it indicates, on the contrary, that glucolysis and glycogenolysis proceed by similar routes. The results of our own experiments reported in this paper show that cell-free extracts of retina convert both glucose and glycogen into lactic acid, but that glucose is more readily attacked. They demonstrate conclusively that the same activators and coenzymes are necessary for both substrates, and that the same enzyme poisons which inhibit glycogenolysis in muscle extracts also prevent glycolysis of both glycogen and glucose in retinal extracts. The intermediary reactions have not been worked out, but the results suggest that phosphorylation and concurrent oxido-reduction of intermediary products are essential reactions in the breakdown of glucose.

After these experiments had been completed Süllman & Vos [1939] reported the preparation of aqueous retinal extracts, and showed that the coenzymes and inhibitors of muscle glycolysis were active. A comparison of our own findings with the results obtained by these authors is included in the discussion.

Methods

Preparation of extract. Ox eyes were brought from the slaughterhouse on ice from 1 to 6 hr. after the animals had been killed. The retinae were dissected out and frozen solid; they were then ground in a cold mortar with sand and an

equal weight of ice-cold glass-distilled water. The mixture was then frozen solid and centrifuged for 20 min.; the supernatant solution was decanted and again frozen and centrifuged for 20 min. This repeated freezing and thawing was intended to assist in breaking up the cells and in promoting extraction. The topmost layer, which appeared perfectly clear after the second spinning, had no glycolytic activity; therefore the whole of the supernatant solution, except the bottom deeply coloured layer, was used after being decanted and thoroughly mixed. No intact cells could be detected in it on microscopic examination. Extracts which are referred to in the text as "fresh extracts" were not dialysed and were used within 4 hr. of their preparation (unless otherwise stated), being kept ice cold during this time.

Dialysis. Cellophane sacs were used, and the extract was dialysed against cold tap water for 6 hr. followed by distilled water in the cold room for approximately another 15 hr. In the earlier experiments running tap water at about 12–14° was used; in later experiments large volumes of tap water at 5°, changed hourly, were used. During the second period of dialysis the distilled water was changed only if the dialysis was continued for more than 24 hr. The use of glass-distilled in place of copper-distilled water made no difference to the results.

Measurement of glycolysis. The extract, together with substrate and other additions, was incubated at 37° in bicarbonate buffer (*p*H 7·4) in an atmosphere of N_2 (95 %) and CO_2 (5 %). 1 ml. of the extract, the substrate and other additions were placed in a Warburg flask containing $NaHCO_3$ (final concentration 0·03 *M*), coenzymes were placed in the side arm of the flask, and the total volume brought to 2 ml. with distilled water. The amounts of the various additions used in each experiment are given in the tables. The flask was then attached to a manometer and shaken in a water bath at 37°. The gas mixture was passed through the flask for 10 min.; the tap was then closed, and a further 5 min. were allowed for equilibrium to be reached, after which the contents of the side arm were tipped in. As no glycolysis took place without the addition of coenzyme, this procedure allowed the precise time at which glycolysis began to be recorded and small differences in equilibrating time were eliminated.

Manometric measurements were usually made as a guide to the rate of glycolysis, but all results reported are in terms of lactic acid formation measured chemically by a modification of the Friedman *et al.* [1927] method. Glycolysis was allowed to continue for 30 min., then trichloroacetic acid was added and lactic acid estimated on an aliquot of the filtrate after precipitation of the sugar with copper and lime.

Estimation of phosphates. The method of Fiske & Subbarow [1925] was used. In order to obtain sufficient filtrate for the determination of both phosphate and lactic acid, all quantities were doubled for these experiments. For the determination of the various acid-soluble phosphate fractions an equal volume of *N* HCl was added to a portion of the protein-free filtrate, aliquots were used for estimation of inorganic and total phosphate, and for inorganic phosphate after hydrolysis in sealed tubes heated in a boiling water bath for 7, 30 or 180 min.

Materials. $MgCl_2$ was used as a source of Mg^{++} throughout. Phosphate was added in the form of the Sørensen mixture (Na_2HPO_4—KH_2PO_4) *p*H 7·4. In one series of experiments a mixture of Na phosphates was substituted, but no difference in the results was obtained, nor did the addition of KCl produce any effect. The Ca salt of adenosinetriphosphate (abbreviated as Ca-ATP) was prepared from cat muscle by the method of Barrenscheen & Filz [1932]. By analogy with the Ba salt (Barrenscheen's formula), the formula of the Ca salt is $C_{10}H_{12}O_{13}N_5P_3Ca_2, 6H_2O$ (theoretical 13·4 % P, found 13·2 %), so that 1 mg.

Ca-ATP is equivalent to 0·73 mg. ATP and very nearly 0·5 mg. adenylic acid (abbreviated AA). For other preparations used we are greatly indebted to a number of biochemists as follows: Dr F. G. Young for glycogen (from mytilus), Dr M. Macfarlane for Ba hexosediphosphate (converted into the Na salt before use), Prof. v. Euler and Dr D. Green for cozymase, Dr Ostern for muscle adenylic acid (used as the Na salt) and Dr Mendel for *dl*-glyceraldehyde.

Glycolysis

Undialysed extract. Cell-free extract of retina can produce lactic acid from glucose, hexosediphosphate or glycogen provided that either ATP or AA is also added. The amount of lactic acid produced from glucose is greater than that

Table 1. *Effects of duration of incubation and of time at which ATP is added on lactic acid formation*

1 ml. extract, 2 mg. Ca-ATP and 4 mg. glucose (or glycogen). Incubated at 37° in 0·03 *M* $NaHCO_3$.

No.	Time of adding Ca-ATP min.	Substrate	Time of adding substrate min.	Lactic acid (mg.) formed after 30 min.	Lactic acid (mg.) formed after 60 min.
7	0	—	—	0·32	0·40
7	30	—	—	0	0·24
4	0	Glucose	0	1·91	1·97
7	0	,,	30	—	0·36
7	30	,,	30	—	1·89
4	0	Glycogen	0	0·79	0·96
7	0	,,	30	—	0·28
7	30	,,	30	—	0·50

obtained from equivalent amounts of the other two substrates (Tables 1 and 4), and the glycolysis proceeds at a more rapid rate. Table 1 shows that lactic acid formation from glucose had almost ceased after 30 min., while that from glycogen was still continuing. This difference in rate was confirmed by manometric readings where it was observed that CO_2 production from glucose fell off continuously during incubation, whilst that from glycogen or hexosediphosphate continued at a nearly uniform rate throughout the experiment. 30 min. was therefore chosen as the standard period for glycolysis. Fig. 1 shows the comparative rates of glycolysis as measured by CO_2 production, using dialysed extract.

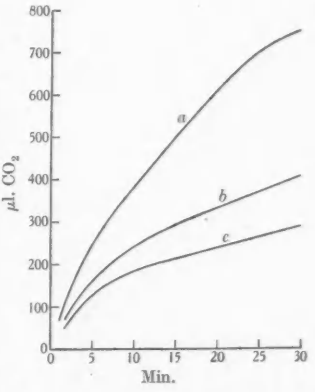


Fig. 1. Manometric measurement of glycolysis. 1 ml. extract, phosphate (0·02 *M*; 0·04 *M* for glycogen), $MgCl_2$ (0·004 *M*), 1 mg. Ca-ATP incubated at 37° in 0·03 *M* $NaHCO_3$. (*a*) 4 mg. glucose, (*b*) 4 mg. hexose equivalent of hexosediphosphate, (*c*) 4 mg. glycogen.

Without addition of substrate very little lactic acid was formed (Tables 1 and 3). This small value has not been subtracted from the total lactic acid production, and the figures in the tables represent total lactic acid formed from added substrate and from carbohydrate present in the extract.

Without addition of ATP or AA there was no glycolysis (Table 6). It was found that ATP was destroyed during incubation with extract when no substrate was

added (Table 1). This destruction must extend beyond the removal of two phosphate radicles since AA is active under certain conditions (Table 6). In the presence of glucose the amount of ATP remaining after 30 min. incubation varied in different extracts (Table 10). It is therefore important that coenzyme should not be added before substrate.

The activity of the extract remained nearly constant during 44 hr. storage in the refrigerator at 5° (Table 2). The activities of different extracts as measured

Table 2. *Effect on lactic acid formation of keeping retinal extract at 5°*

1 ml. extract, 2 mg. Ca-ATP, 4 mg. glucose (or glycogen). Incubated for 30 min. at 37° in 0·03 *M* $NaHCO_3$.

No.	Hr. after preparation	Lactic acid (mg.) formed from: Glucose	Glycogen
6	0	1·80	0·66
	2·5	1·64	0·47
	6	1·53	0·60
	20	1·77	0·54
	44	1·61	0·65
	92	0·53	0·50
7	0	1·89	—
	20	1·78	—
20	0	3·94*	—
	3	4·04*	—

* Phosphate (0·01 *M*) and $MgCl_2$ (0·004 *M*) also added.

Table 3. *Lactic acid formation from different extracts*

1 ml. extract, 2 mg. Ca-ATP, incubated for 30 min. at 37° in 0·03 *M* $NaHCO_3$.

No.	Lactic acid (mg.) formed from: No added substrate	4 mg. glucose	4 mg. glycogen
3	—	2·00	0·56
4	0·30	1·91	0·79
5	0·17	1·61	0·49
6	0·30	1·80	0·66
7	0·32	1·78	—
8	0·41	1·73	0·70
10	0·32	1·74	0·67
15	—	1·64	—
17	—	1·62	—

by lactic acid production (Table 3) were sufficiently constant for typical results to be selected in compiling the tables illustrating the behaviour of the extract in the presence of phosphate, Mg and ATP. Each point noted was, of course, confirmed by experiments with a number of different extracts.

The amounts of lactic acid produced from glucose and from glycogen by fresh extract in the presence of ATP were increased by addition of phosphate (Table 4). The yield of lactic acid from glucose was maximal when sufficient phosphate was added to give a concentration between 0·01 *M* and 0·02 *M*. The final concentration in the mixture was slightly higher than that produced by the added phosphate owing to the presence of a small amount of phosphate in the extract. With higher concentrations of phosphate (0·08 *M*), glucolysis was inhibited to some extent. With glycogen maximal glycolysis occurred with a higher phosphate concentration (0·05 *M*) and inhibition at a concentration of 0·08 *M* was slight.

Table 4. *Effect of alteration of phosphate concentration on lactic acid formation*

1 ml. extract, 4 mg. glucose, glycogen or hexose equivalent of hexosediphosphate, incubated for 30 min. at 37° in 0·03 *M* $NaHCO_3$.

		Additions		Lactic acid (mg.) formed with phosphate concentrations (*M*) of								
No.	Substrate	Ca-ATP mg.	$MgCl_2$ *M*	0	0·001	0·002	0·01	0·02	0·04	0·05	0·06	0·08
				Fresh extract								
8	—	2	0	0·41	—	—	0·56	—	—	0·67	—	0·57
8	Glucose	2	0	1·73	—	—	2·86	2·80	—	2·52	—	2·28
8	Glycogen	2	0	0·70	—	—	—	1·08	—	1·15	—	1·04
15	Hexosediphosphate	2	0	1·09	—	—	1·04	—	—	—	—	—
				Dialysed extract								
18	—	2	0·004	0·17	—	—	—	0·19	—	—	—	—
13	Glucose	1	0·004	0·23	—	1·23	2·16	2·62	2·52	—	2·64	—
17	„	2	0·004	0·86	1·24	—	2·80	3·06	3·20	—	2·96	—
13	Glycogen	1	0·004	0·04	—	0·23	0·31	0·53	0·65	—	0·67	—
12	„	2	0·02	0·10	—	0·23	0·64	0·91	1·08	—	—	—
14	Hexosediphosphate	1	0·004	1·22	—	1·33	1·37	1·48	1·36	1·35	—	—

Note. The concentrations stated are the final concentrations which would be produced by the amounts of phosphate or $MgCl_2$ added in each case. In the fresh extract, the actual concentrations are slightly higher, owing to the presence of small amounts of these substances in the extract.

Table 5. *Effect of alteration of the concentration of $MgCl_2$ on lactic acid formation*

1 ml. extract, 4 mg. glucose, glycogen or hexose equivalent of hexosediphosphate, incubated for 30 min. at 37° in 0·03 *M* $NaHCO_3$.

		Additions		Lactic acid (mg.) formed with $MgCl_2$ concentrations (*M*) of						
No.	Substrate	Ca-ATP mg.	Phosphate *M*	0	0·0004	0·001	0·004	0·01	0·02	0·04
				Fresh extract						
17	Glucose	2	0·01	2·66	—	—	—	—	3·49	3·02
15	Hexosediphosphate	2	0·01	1·04	—	—	—	—	2·17	—
				Dialysed extract						
17	Glucose	2	0·02	0·55	1·61	2·43	3·09	2·89	—	2·15
14	Glycogen	1	0·04	0·60	0·76	0·80	0·79	0·71	0·43	—
14	Hexosediphosphate	1	0·02	0·91	1·15	1·44	1·48	1·43	1·30	—

See note to Table 4.

In the presence of optimal concentrations of phosphate, the addition of $MgCl_2$ caused a further increase in lactic acid production from glucose. With hexosediphosphate as substrate the addition of phosphate caused no increase in lactic acid production, but the addition of $MgCl_2$ caused a considerable increase (Tables 4 and 5).

In fresh extracts AA could replace ATP as coenzyme (Table 6). In the absence of added phosphate the amount of lactic acid formed from glucose was less with AA than with ATP, but in the presence of optimal concentrations of phosphate the amount of lactic acid formed was greater if AA were used. With hexosediphosphate AA was always more effective, the presence or absence of added phosphate making little difference. With glycogen it was shown that AA could replace ATP, but the relative amounts of lactic acid formed and the influence of phosphate were not thoroughly investigated.

Table 6. *Effect of alteration of quantity of Ca-ATP (or AA) on lactic acid formation*

1 ml. extract, 4 mg. glucose, glycogen or hexose equivalent of hexosediphosphate, incubated for 30 min. at 37° in 0·03 *M* $NaHCO_3$.

		Additions		Lactic acid (mg.) formed with added										
				Ca-ATP (mg.)							AA (mg.)			
No.	Substrate	Phosphate *M*	$MgCl_2$ *M*	0	0·01	0·1	0·5	1·0	2·0	5·0	0·5	1·0	2·0	2·5
				Fresh extract										
5	—	0	0	0	—	—	—	—	0·17	—	—	0·12	0·25	—
12	—	0·01	0	—	—	—	—	0·46	0·37	0·26	0·19	0·49	—	0·46
5	Glucose	0	0	0	—	—	—	—	1·61	—	—	1·21	1·44	—
12	,,	0·01	0	—	—	—	—	2·74	2·69	1·86	3·14	3·14	—	3·22
5	Glycogen	0	0	—	—	—	—	—	0·49	—	—	0·28	0·80	—
15	Hexosediphosphate	0	0	—	—	—	—	—	1·09	—	—	1·46	—	—
15	,,	0·01	0	—	—	—	—	—	1·04	—	—	1·47	—	—
				Dialysed extract										
18	—	0·01	0·004	0	—	—	—	—	0·19	—	—	—	—	—
8	Glucose	0·02	0·02	—	—	—	—	—	2·60	—	—	0·63	1·17	—
13	,,	0·02	0·004	—	—	—	—	2·62	—	—	0·05	—	—	—
16	,,	0·02	0·004	0	0	0·12	2·52	2·94	3·34	3·22	—	—	—	—
13	Glycogen	0·04	0·004	—	—	—	—	0·65	—	—	0·30	—	—	—
16	,,	0·04	0·004	0	0	—	0·31	0·54	0·72	0·85	—	—	—	—
16	Hexosediphosphate	0·02	0·004	0·23	0·20	0·53	1·32	1·69	2·06	2·48	—	—	—	—

Note. 2 mg. Ca-ATP≡1 mg. AA. See note to Table 4.

Table 7. *Effect of adding cozymase on lactic acid formation*

1 ml. extract, 4 mg. glucose, glycogen or hexose equivalent of hexosediphosphate, incubated for 30 min. at 37° in 0·03 *M* $NaHCO_3$. For dialysed extract 0·004 *M* $MgCl_2$ and 0·02 *M* phosphate (0·04 *M* for glycogen) also added.

Additions			Lactic acid (mg.) formed from			
Ca-ATP mg.	Cozymase mg.	Dialysis hr.	No added substrate	Glucose	Glycogen	Hexosediphosphate
			Fresh extract			
2	0	—	—	1·77	—	—
0	0·2	—	—	0·37	—	—
2	0·2	—	—	1·74	—	—
			Dialysed extract			
2	0	20	—	2·86	0·82	1·64
0	2	20	—	0	0	0·26
2	2	20	0·19	2·97	0·86	1·56
2	0	48	—	2·25	0·49	1·34
2	2	48	—	2·86	0·51	1·41

ATP could not be replaced by cozymase for inducing glycolysis in fresh extracts, nor did the addition of cozymase in the presence of ATP increase the yield of lactic acid (Table 7).

The effect of dialysis. After the extract had been dialysed against cold water for 20 hr. addition of coenzyme (ATP) only was not sufficient to produce glycolysis. In order that glucose might be converted into lactic acid it was necessary to add both phosphate and $MgCl_2$. The concentrations of these substances required for producing maximal glycolysis have been determined and are recorded in Tables 4 and 5.

When hexosediphosphate was used as substrate the addition of phosphate produced only a small increase in glycolysis, whilst the addition of $MgCl_2$ greatly increased lactic acid production. With glycogen, on the other hand, $MgCl_2$ had little effect, but it was essential to add phosphate; increasing concentrations gave greater yields of lactic acid and no inhibitory effect was observed with the highest concentration (0·06 *M*) used.

Without the addition of ATP or AA there was no glycolysis of either glucose or glycogen, but a trace of lactic acid was produced when hexosediphosphate was incubated with extract (Table 6). The effect of substituting AA for ATP was tried in only two experiments with dialysed extract. In one case, extract no. 8, AA produced some activity when added together with phosphate and $MgCl_2$, but extract no. 13, which showed normal activity with ATP, was quite inactive when an equivalent amount of AA was substituted. The only difference in the preparation of these two extracts recorded was in the temperature of the tap water during the preliminary dialysis. In the case of extract no. 8 the temperature did not rise above 12°, but with extract no. 13 it had risen to 18° at the end of 6 hr. The possible bearing of this on the observed result is discussed later.

Cozymase could not replace ATP when added to extract dialysed for 20 hr. (Table 7). When it was added together with ATP it caused no increase in lactic acid formation. But, in the case of extract which had been dialysed for 48 hr. and which showed a diminution of glycolytic activity in the presence of ATP, the addition of cozymase caused a definite increase in lactic acid production from glucose. The increases obtained in the case of hexosediphosphate and glycogen were not significant. It is possible that cozymase is removed from retinal extract only after prolonged dialysis. Sym *et al.* [1930] have shown that retina contains large amounts of cozymase and Green *et al.* [1937] state that cozymase associated with triosephosphate mutase is not completely removed by dialysis.

Effect of inhibitors. Glycolysis of all substrates was inhibited by addition of iodoacetate (IAA), NaF or glyceraldehyde (Table 8). A concentration of *M*/20,000 IAA inhibited lactic acid production from glucose almost completely, from glycogen by about 50 %, and from hexosediphosphate hardly at all, while a tenfold increase in concentration (*M*/2000) effectively inhibited glycolysis

Table 8. *Inhibition of glycolysis*

1 ml. extract, 2 mg. Ca-ATP, 4 mg. glucose, glycogen or hexose equivalent of hexosediphosphate, incubated for 30 min. at 37° in 0·03 *M* $NaHCO_3$. For extracts nos. 20 and 21 phosphate, to give a concentration of 0·01 *M*, and $MgCl_2$, to give a concentration of 0·004 *M*, were also added.

No.	Inhibitor	Lactic acid (mg.) formed from: Glucose	Glycogen	Hexosediphosphate
	Fresh extract			
10	—	1·74	0·67	1·25
10	*M*/20,000 IAA	0·15	0·29	1·15
10	*M*/2000 IAA	0·02	0·01	0·19
10	*M*/20 NaF	0·01	0	0·25
20	—	4·04	—	—
20	*M*/50 NaF	0·04	—	—
	Dialysed extract			
21	—	3·78	1·38	2·20
21	*M*/500 *dl*-glyceraldehyde	3·59	1·38	2·12
21	*M*/5 *dl*-glyceraldehyde	0·63	0·22	0·50

from all substrates. NaF was effective in a concentration of $M/20$ for all substrates and also at $M/50$ for glucose; the effect of the smaller concentration was not determined with the other substrates.

Dialysed extracts were used for testing the effect of glyceraldehyde, since Boyland & Boyland [1938] have shown that undialysed muscle extract contains a substance which will neutralize its inhibitory effect. It was found necessary to use concentrations as high as $M/10$ *l*-glyceraldehyde (added as $M/5$ *dl*-glyceraldehyde) before appreciable inhibition was induced. With this inhibitor retinal extract behaves in a manner similar to muscle extract and is not analogous to the tumour tissue studied by Mendel [Mendel, 1929; Mendel *et al.* 1938] where glycolysis could be inhibited by very much lower concentrations of *l*-glyceraldehyde. Süllman & Vos [1939] found glyceraldehyde to be inhibitory in rather lower concentrations than those which were effective in our experiments, but in neither case was inhibition quite complete.

Formation of phosphoric esters

The foregoing results prove that the presence of phosphate is necessary for the production of lactic acid from glucose or from glycogen, but they give no indication of the part played by the phosphate ion. In the case of muscle extract it has been shown that a series of carbohydrate-phosphate esters are formed, and, if the same enzyme system were present in retinal extract, the formation of the same esters might be expected to occur during glycolysis. We have attempted to obtain evidence of the formation of phosphoric esters by determining the inorganic phosphate and the organic phosphate fractions before and after incubation, and also after incubation of the mixture in the presence of fluoride.

By observing the rate of hydrolysis in $N/2$ HCl at 100°, although it is not possible to characterize each ester in the mixture, evidence of an accumulation of esterified phosphate or of the transfer of phosphate from the easily hydrolysable ATP to the more difficultly hydrolysable carbohydrate-phosphate esters can be obtained.

For all these experiments $MgCl_2$, phosphate and coenzyme (either ATP or AA) were added to fresh extract.

Initial values. Each of the two extracts studied contained inorganic phosphate and some difficultly hydrolysable ester. A little ATP (i.e. phosphoric ester hydrolysed in 7 min.) was present in extract no. 20, but none in extract no. 19 (Table 9).

Table 9. *Acid-soluble phosphate esters of retinal extract*

Values represent mg. inorganic P in 2 ml. extract before and after hydrolysis in $N/2$ HCl. Suffixes denote time of hydrolysis in min.; P_t, total phosphate.

No.	P_0	P_7-P_0	P_{30}-P_7	P_{180}-P_7	P_t-P_{180}
19	0·29	0	0·08	0·06	0·19
20	0·17	0·10	−0·04	0·03	0·21

Glucose as substrate. After incubation of glucose with ATP as coenzyme there was no decrease in inorganic phosphate. In one experiment (Table 10, [1]) it hardly changed in amount, in another (Table 10, [2]) it increased. In most experiments in which AA was used instead of ATP there was some decrease in inorganic phosphate, but this decrease was accounted for by gain in ATP, that is in the fraction hydrolysed in 7 min. (Table 10, [6] and [7]). In no case was any accumulation of phosphoric ester with a longer hydrolysis time observed.

Table 10. *Rate of hydrolysis of acid-soluble phosphate esters before and after incubation*

2 ml. fresh extract, substrate, phosphate (0·01 *M*), $MgCl_2$ (0·004 *M*) and other additions as stated, incubated for 30 min. at 37° in 0·03 *M* $NaHCO_3$, final volume 4 ml. Concentration of NaF, 0·02 *M*; of IAA, *M*/2000. Values represent mg. inorganic P before and after hydrolysis in *N*/2 HCl for 2 ml. extract plus additions. Suffixes denote time of hydrolysis, P_t = total phosphate.

No.		P_0	P_7-P_0	$P_{30}-P_7$	$P_{180}-P_{30}$	P_t-P_{180}	Lactic acid mg.
	Extract 19 + 4 mg. Ca-ATP						
	Before incubation	1·59	0·29	0·09	0·17	0·21	—
1	After incubation + 8 mg. glucose	1·58	0·24	0·11	0·13	0·22	7·01
	Extract 20 + 4 mg. Ca-ATP						
	Before incubation	1·46	0·42	−0·01	0·11	0·30	—
2	After incubation + 8 mg. glucose	1·82	0·15	0	0·15	0·31	7·87
3	After incubation + 8 mg. glucose + NaF	1·41	0·10	0·03	0·01	0·94	0·08
4	After incubation + 8 mg. pyruvate + NaF	1·44	0·15	−0·02	0·06	0·79	1·75
5	After incubation + 8 mg. pyruvate + 8 mg. glucose + NaF	0·39	0·18	0·07	0·18	1·58	3·96
	Extract 19 + 2 mg. AA						
	Before incubation	1·50	0	0·12	0·11	0·25	—
6	After incubation + 8 mg. glucose	1·32	0·22	0·02	0·26	0·05	7·67
	Extract 20 + 2 mg. AA						
	Before incubation	1·45	0·10	−0·03	0·10	0·30	—
7	After incubation + 8 mg. glucose	1·53	0·19	0	0·30	−0·05	8·01
8	After incubation + 8 mg. glucose + NaF	1·51	0·08	0·11	−0·11	0·45	0·03
9	After incubation + 8 mg. pyruvate + NaF	1·10	0·14	−0·06	0·11	0·71	1·62
10	After incubation + 8 mg. pyruvate + 8 mg. glucose + NaF	0·43	0·13	−0·01	0·13	1·29	3·62
	Extract 11 + 4 mg. Ca-ATP						
	Before incubation	2·00	0·28	0·50	0·40	0	—
11	After incubation + 8 mg. glucose + IAA	2·32	0·04	0·30	0·34	0	0·06
	Extract 20 + 4 mg. Ca-ATP + hexosediphosphate (≡ 6·3 mg. hexose)						
	Before incubation	1·52	1·20	0·31	0·95	0·36	—
12	After incubation	3·38	0·36	0·06	0·04	0·74	5·12
13	After incubation + 8 mg. glucose	2·04	0·86	0·40	0·30	0·76	8·86
14	After incubation + 8 mg. pyruvate + NaF	1·24	0·62	0·32	0·35	2·00	4·95
15	After incubation + 8 mg. pyruvate + 8 mg. glucose + NaF	0·50	0·83	0·48	0·59	2·02	5·22
	Extract 19 + 2 mg. AA + hexosediphosphate (≡ 6·8 mg. hexose)						
	Before incubation	1·64	0·91	0·58	0·98	0·21	—
16	After incubation	2·83	0·51	0·23	0·10	0·48	4·80

See note to Table 4.

Hexosediphosphate as substrate. After incubation of the extract with hexosediphosphate there was a large increase in inorganic phosphate and an increase in difficultly hydrolysable ester, i.e. ester not hydrolysed in 180 min. (Table 10, [12] and [16]). The amount of lactic acid formed was approximately that calculated as equivalent to the increase in inorganic phosphate.

Hexosediphosphate plus glucose as substrate. When a mixture of approximately equivalent amounts of glucose and hexosediphosphate was incubated with extract (Table 10, [13]), the yield of lactic acid (8·86 mg.) was a little greater than that from the same amount of glucose alone (7·87 mg.), but not nearly so great as the sum of the amounts formed when the two substrates were incubated separately (7·87 + 5·12 mg., Table 10). About one-third of the hexosediphosphate remained unchanged after incubation and some ester largely hydrolysed in 30 min. was formed. Inorganic phosphate increased and it was not possible to

decide whether the added glucose was esterified and the lactic acid derived entirely from hexosediphosphate or whether the glucose was converted into lactic acid directly. However, the results indicate that the same enzymes are concerned in the conversion of both glucose and hexosediphosphate into lactic acid, for if there were two entirely different mechanisms operating one might expect that the total lactic acid formed from the mixture would be equal to the sum of the amounts formed from each substrate separately.

Glucose as substrate in the presence of NaF. When glucose was incubated with NaF-poisoned extract no lactic acid was formed, added ATP disappeared and there was a considerable formation of difficultly hydrolysable ester (Table 10, [3] and [8]).

Incubation of glucose with pyruvate in the presence of NaF. It has been suggested that in yeast extract and in blood corpuscles glucose is phosphorylated concurrently with oxidation of triosephosphate ester. In a system poisoned with NaF, any phosphoglycerate which may be formed cannot be converted into pyruvic acid, which acts as acceptor for hydrogen during oxidation of triosephosphate; therefore, the formation of lactic acid is inhibited. If a similar oxidation-reduction system is present in retinal extract, a fluoride-poisoned extract should be able to form lactic acid from glucose provided that pyruvate is also added, and at the same time there should be an increase in phosphoric ester. This was found to be the case (Table 10, [5] and [10]). The esterified phosphate was found in the fraction not hydrolysed in 180 min. The increase in phosphate ester was balanced by a decrease in inorganic phosphate and ATP phosphate, if ATP were used as coenzyme; if AA were used all of the esterified phosphate was derived from inorganic phosphate.

Incubation of pyruvate alone in the presence of NaF. When pyruvate without glucose was incubated with fluoride-poisoned extract there was a considerable formation of lactic acid and some increase in difficultly hydrolysable phosphate ester (Table 10, [4] and [9]). As in the presence of glucose, inorganic phosphate was esterified if AA was used as coenzyme, but phosphate was transferred from ATP if it was present. The extracts used for these experiments were not dialysed and therefore probably contained some carbohydrate which could be esterified and possibly some other substrate which could be oxidized concurrently with reduction of pyruvate to lactate.

Incubation of hexosediphosphate with pyruvate in the presence of NaF. When hexosediphosphate was incubated with pyruvate in fluoride-poisoned extract

Table 11

Comparison of increase in phosphate ester unhydrolysed in 180 min. with that calculated from the lactic acid formation (on the assumption that 1 mol. of lactic acid is equivalent to 1 mol. of phosphate), in extract incubated with NaF and pyruvate. For experimental details see Table 10.

No. in Table 10	Substrate	Coenzyme	Decrease in inorganic phosphate mg.	Increase in ester-P (mg.)		Lactic acid formed mg.
				Found	Calc.	
4	—	Ca-ATP	0·02	0·49	0·60	1·75
9	—	AA	0·35	0·41	0·56	1·62
5	Glucose	Ca-ATP	1·07	1·28	1·36	3·96
10	,,	AA	1·02	0·99	1·25	3·62
14	Hexosediphosphate	Ca-ATP	0·28	1·64	1·70	4·95
15	Glucose + hexosediphosphate	Ca-ATP	1·02	1·66	1·80	5·22

(Table 10, [14]) it was not dephosphorylated but was converted into an ester not hydrolysed in 180 min. The increase in this fraction was approximately equivalent to the lactic acid formed (Table 11). At the same time some inorganic phosphate was esterified.

Incubation of glucose plus hexosediphosphate with pyruvate in the presence of NaF. If glucose, hexosediphosphate and pyruvate were incubated together in fluoride-poisoned extract (Table 10, [15]) there was a decrease in inorganic phosphate, but the increase in difficultly hydrolysable ester greatly exceeded the decrease in phosphate; the amount formed was almost the same as when hexosediphosphate and pyruvate were incubated alone under the same conditions.

Incubation of extract poisoned with IAA. When glucose was incubated with extract poisoned with IAA there was no esterification of phosphate or transference of phosphate from ATP (Table 10, [11]).

Discussion

According to the present conception of the mechanism of anaerobic carbohydrate breakdown the three most important steps are: (1) phosphorylation, leading to formation of triosephosphate; (2) oxidation of triosephosphate to phosphoglycerate with concurrent reduction of either a molecule of pyruvate or another molecule of triosephosphate; (3) dehydration and dephosphorylation of phosphoglycerate to form pyruvate, which, after the induction period, supplies the main reductant in the second step.

The results reported in this paper support the hypothesis that this system is present in retinal extract. Not only are the same activators and coenzymes essential but fluoride inhibition of glycolysis can be reversed by addition of pyruvate, lactic acid formation then being accompanied by formation of a difficultly hydrolysable phosphoric ester.

The initial step in the process, phosphorylation of carbohydrate, may occur in three ways, by direct phosphorolysis, by phosphorylation coupled with either oxidoreduction or with dephosphorylation of phosphopyruvate. So far as is known the first method is applicable only to polysaccharides, when phosphate is added directly to the molecule as the glucosidic linkages are opened. The second and third methods may result in phosphorylation of glucose, glycogen, hexosemonophosphate or creatine, and the necessary enzyme systems have been found in various tissue extracts.

The inability to detect phosphoric esters during glucose breakdown does not necessarily mean that such esters are not formed; for only if the rate of formation is greater than the rate of breakdown will a measurable quantity of phosphoric esters accumulate. When the extract was poisoned with NaF, although there was no decrease in inorganic phosphate, there was an increase in difficultly hydrolysable phosphoric ester at the expense of ATP phosphate. If pyruvate was added together with glucose in fluoride-poisoned extract there was a large decrease in inorganic phosphate and a corresponding increase in difficultly hydrolysable ester. This can be explained on the assumption that pyruvate is reduced to lactate while phosphoric ester is oxidized to the difficultly hydrolysable phosphoglycerate and at the same time inorganic phosphate is esterified. If the esterified phosphate remained as hexosemonophosphate one would expect that for every molecule of lactic acid formed two molecules of phosphate would appear as difficultly hydrolysable ester. In fact not quite one molecule accumulated per molecule of lactic acid formed (Table 11); therefore, we conclude that the difficultly hydrolysable ester is phosphoglycerate, formed by the oxidation

of the phosphoric ester first formed. Further evidence that phosphorylation of glucose is accompanied by oxido-reduction is provided by the experiment on an extract poisoned with IAA (known to inhibit the oxido-reduction system), in which no phosphorylation at all occurred.

In the presence of fluoride and pyruvate lactic acid formation from glucose was only about half as great as in unpoisoned extract, suggesting that normally glucose esterification is accomplished by an additional route, namely, by transference of phosphate from phosphopyruvate. Increase in phosphate concentration may be expected to aid phosphorylation until a point is reached at which further increase inhibits dephosphorylation of phosphopyruvate [see Lennerstrand, 1936], so that the finding of an optimum phosphate concentration is explained.

Since glycolysis took place in dialysed extract on addition of coenzyme, $MgCl_2$ and phosphate, some oxidizable or phosphorylated substrate must still have been present. Our extracts were not shaken or stirred during dialysis, so that it is quite possible that traces of substrate, especially if they were associated with protein, would not be removed.

In fresh extract addition of both glucose and hexosediphosphate resulted in a slightly larger formation of lactic acid than addition of glucose alone, probably because glycolysis proceeded at the maximal rate from the beginning of incubation. This experiment affords strong evidence for supposing that the same system is concerned in breakdown of both glucose and hexosediphosphate, for if the two substrates are attacked by entirely different enzymes the amount of lactic acid formed when both are incubated together should approximate to the sum of that formed from the two incubated separately.

The results reported here do not settle the question of which form of nucleotide is active in retinal extract. For oxido-reduction in brain extract Meyerhof [1938] found that only adenosinediphosphate was active; in retinal extract adenosinediphosphate may well be formed from either ATP or AA. ATP was more active than AA in promoting glycolysis in low phosphate concentrations, but when the phosphate concentration was optimal, AA gave better results. If phosphate were split off from ATP the actual phosphate concentration of the extract would be increased, so that if the initial concentration were low the glycolytic rate would be increased, but at higher concentrations the optimum might be exceeded.

When AA was used as the coenzyme there was always a decrease in inorganic phosphate and formation of ATP (or adenosinediphosphate). If this phosphorylation is independent of glycolysis, then the difference in behaviour of extracts nos. 8 and 13 (Table 6) may be explained by supposing that the phosphatese was destroyed by the higher temperature (18°) reached during dialysis of extract no. 13, so that the active form of the nucleotide could not be formed from added AA. Süllman & Vos [1939] found that AA could no longer activate their extracts after dialysis for 24 hr.

Lactic acid formation from hexosediphosphate was less than from glucose, and was accompanied by formation of difficultly hydrolysable ester; the reason for this may be that dephosphorylation is disturbed by lack of a phosphate acceptor. ATP can activate the breakdown of hexosediphosphate in dialysed extract; therefore, either it is dephosphorylated to provide an acceptor, or oxido-reduction can proceed at a reduced rate without concurrent phosphorylation. Furthermore, when hexosediphosphate was incubated with pyruvate in fluoride-poisoned extract both lactic acid and difficultly hydrolysable ester formation greatly exceeded esterification of inorganic phosphate (Table 11). This suggests that, either oxido-reduction takes place without concurrent phosphorylation or

the phosphorylated product is immediately dephosphorylated. The activating effect of Mg^{++} on hexosediphosphate breakdown is to be expected as Lohmann [1933] has shown that Mg is necessary for its dephosphorylation.

The slow rate of glycogenolysis may be due to lack of the enzymes necessary for the initial phosphorolysis, since Süllman & Vos [1939] found that glucose-1-phosphate is attacked nearly as readily as glucose by retinal extract. Increase in phosphate concentration would be expected to aid phosphorolysis, and the small activating effect of Mg^{++} may be due to the fact that it is not essential for this first step in breakdown of glycogen [Cori *et al.* 1938].

The results of Süllman & Vos [1939] on glycolysis in retinal extract are in general agreement with those reported here, and they also support the hypothesis that carbohydrate breakdown proceeds according to the Meyerhof-Embden scheme. These authors used AA as coenzyme in all their experiments but found that it was necessary to add phosphate, some of which was esterified during incubation. They report that many of their extracts were active without addition of coenzyme; none of our preparations which were tested in this way showed any activity unless ATP or AA were added. Probably, in our preparation, nucleotide originally present in the cells was destroyed by a nucleotidase extracted along with the other enzymes; a similar explanation would account for the destruction of added ATP (Table 1). Reis [1934] and Gulland & Jackson [1938] have found a very active nucleotidase in retina. Süllman & Vos did not freeze the retinae during preparation of their extracts, so that extraction of cell contents may have been less complete and in some cases insufficient nucleotidase extracted to destroy all the nucleotide before incubation. The same explanation, namely, incomplete extraction, would account for their finding that addition of cozymase and AA increased glucolytic activity more than addition of AA alone.

It is difficult to make a direct comparison between the activities of our extracts and those of Süllman & Vos, because in their work the conditions, particularly of time of incubation, were not standard throughout. If lactic acid production had almost ceased after 30 min. incubation, as in our experiments, then their extracts were, with one exception, rather less active than ours; on the other hand, if lactic acid production continued during the whole of their incubation periods, then their extracts were very much less active.

Summary

1. An aqueous extract of ox retina which on addition of ATP or AA converted added glucose, glycogen or hexosediphosphate into lactic acid was prepared.

2. The amount of lactic acid formed from glucose was increased by the addition of phosphate and $MgCl_2$; the yield from glycogen was increased by phosphate, but not by $MgCl_2$; and the yield from hexosediphosphate was increased by $MgCl_2$, but not by phosphate.

3. After the extract had been dialysed for 20 hr. no lactic acid was formed from glucose unless phosphate and $MgCl_2$, as well as ATP, were added; with glycogen as substrate it was necessary to add phosphate; and with hexosediphosphate $MgCl_2$ without phosphate produced activity.

4. Adenylic acid could replace ATP as coenzyme, but with glucose as substrate the amount of lactic acid formed was less; if, however, phosphate was also added, a higher yield of lactic acid was obtained with AA. With hexosediphosphate AA was always more effective.

5. Cozymase could not take the place of ATP or AA as coenzyme. When added in addition to ATP it did not increase glycolysis in fresh or in 20 hr.-dialysed extract, but with extracts which had been dialysed for 48 hr. the addition of cozymase in the presence of ATP increased the yield of lactic acid from glucose.

6. Glycolysis of all three substrates was inhibited by addition of IAA, NaF or glyceraldehyde. In fluoride-poisoned extract, addition of pyruvate caused formation of lactic acid.

7. In unpoisoned extract incubated with glucose no evidence of the formation of carbohydrate-phosphoric esters could be obtained. When AA was used as coenzyme there was a transference of inorganic phosphate to an easily hydrolysed ester, probably ATP. When hexosediphosphate was used as substrate it was almost all broken down, there was a large increase in inorganic phosphate and some formation of a difficultly hydrolysable ester.

8. In fluoride-poisoned extract there was some formation of difficultly hydrolysable ester when glucose was used as substrate; when pyruvate was added in addition to glucose this formation of difficultly hydrolysable ester was greatly increased and was approximately equivalent to the amount of lactic acid formed, and to the decrease in inorganic phosphate.

9. Incubation of glucose and hexosediphosphate together yielded only a slightly larger amount of lactic acid than incubation of glucose alone.

The expenses of this work have been borne by a grant from the Medical Research Council; one of us (M. C. B.) is also indebted to the Council for a personal grant. Our grateful thanks are due to those biochemists who assisted us with gifts of material. It is a pleasure to record our thanks to Miss Marta Rousseau for her help with some of the chemical estimations.

REFERENCES

Barrenscheen & Filz (1932). *Biochem. Z.* **250**, 281.
Boyland & Boyland (1938). *Biochem. J.* **32**, 321.
Bumm & Fehrenbach (1931). *Hoppe-Seyl. Z.* **195**, 101.
Cori, Colowick & Cori (1938). *J. biol. Chem.* **124**, 543.
Euler, Günther & Vestin (1936). *Hoppe-Seyl. Z.* **240**, 265.
Fiske & Subbarow (1925). *J. biol. Chem.* **66**, 375.
Friedman, Cotonio & Shaffer (1927). *J. biol. Chem.* **73**, 335.
Green, Needham & Dewan (1937). *Biochem. J.* **31**, 2327.
Gulland & Jackson (1938). *Biochem. J.* **32**, 597.
Kerly & Bourne (1939). *J. Soc. chem. Ind.* **58**, 254.
Lennerstrand (1936). *Biochem. Z.* **289**, 104.
Lohmann (1933). *Biochem. Z.* **262**, 137.
Mendel (1929). *Klin. Wschr.* **8**, 169.
—— Strelitz & Mundel (1938). *Science*, **88**, 149.
Meyerhof (1938). *Bull. Soc. Chim. biol., Paris*, **20**, 1335.
Needham & Lehmann (1937). *Biochem. J.* **31**, 1210.
Reis (1934). *Bull. Soc. Chim. biol., Paris*, **16**, 385.
Süllman (1937). *Arch. Augenheilk.* **110**, 303.
—— & Vos (1939). *Enzymologia*, **6**, 246.
Sym, Nillson & Euler (1930). *Hoppe-Seyl. Z.* **190**, 228.
Warburg, Posener & Negelein (1924). *Biochem. Z.* **152**, 309.

69. THE CONSTITUTION OF CARVIOLIN: A COLOURING MATTER OF *PENICILLIUM CARMINO-VIOLACEUM* BIOURGE

By H. G. HIND

University College, Nottingham, and the Lister Institute, London

(*Received 15 February 1940*)

It has recently been shown [Hind, 1940] that *P. carmino-violaceum* Biourge produces two pigments, which were named carviolin ($C_{16}H_{12}O_6$) and carviolacin ($C_{20}H_{16}O_7$). These compounds are polyhydroxyanthraquinones and each contains a methyl ether group.

From a study of the properties of carviolin, and from analogy with other mould anthraquinones [Raistrick, 1938] this compound appeared to be a methyl ether of a tetrahydroxy-β-methylanthraquinone. Further examination of the chemical properties of the pigment has in fact, revealed that it is a methyl ether of ω-hydroxyemodin. This conclusion was arrived at from a consideration of the following properties of carviolin.

(1) It is well known that oxidation of α-hydroxyanthraquinones with Pb_3O_4 or MnO_2 in conc. H_2SO_4, followed by reduction of the product in aqueous solution with SO_2, introduces a hydroxyl group in the *p*-position to the α-hydroxyl group already in the molecule. Treatment of carviolin after this manner gave a compound having the absorption bands of a 1:4:5:8-tetrahydroxyanthraquinone. This result could only be expected from a compound having α-hydroxyl groups in both benzene nuclei. In view of the fact that all mould anthraquinones so far identified have been derivatives of 1:8-dihydroxyanthraquinone, it was thought probable that this might also apply to carviolin.

(2) Treatment with HBr in boiling glacial acetic acid solution gave a monobromo derivative of the demethylated pigment. The bromine atom was removed from the molecule by boiling for a short time with aqueous silver acetate, indicating the presence of a $-CH_2OH$ or a $-CH_2OMe$ grouping in the carviolin molecule. It is a necessary corollary of (1) that this grouping must be in the β-position.

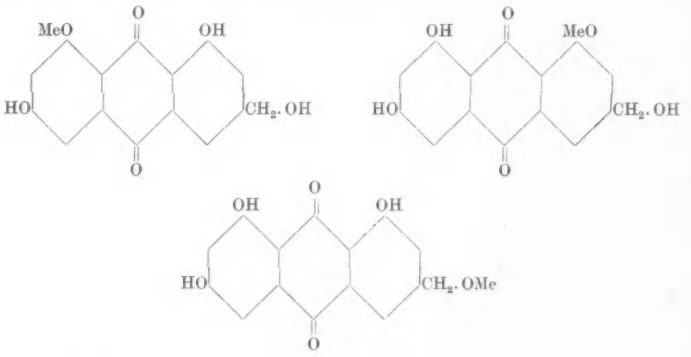
(3) Since carviolin is soluble in cold carbonate solution it has probably a free β-hydroxyl group. Dyeing tests with demethylated carviolin showed that this was not a derivative of alizarin and the methoxyl was not therefore adjacent to one of the α-hydroxyl groups.

(4) The absorption curves for carviolin in alcoholic acid and alkali were similar to those of *Frangula*-emodin in the same solvents.

These properties indicated that the demethylated pigment might be identical with ω-hydroxyemodin.

Through the kindness of Prof. Raistrick it has been possible to make a direct comparison of ω-hydroxyemodin and carviolin. These compounds yield identical

fully methylated methyl ethers. Allowing for the free β-hydroxyl group, carviolin must have one of the following three structures.



Experimental

Demethylation of carviolin

Demethylation of carviolin by heating with conc. H_2SO_4 was unsatisfactory; much resinification occurred at 150°. The demethylation was successfully carried out by refluxing with HBr and glacial acetic acid; this method was found to be satisfactory for demethylation of methoxyanthraquinones by Graves & Adams [1923].

Carviolin (100 mg.) was refluxed with HBr (constant-boiling, 15 ml.) and glacial acetic acid (10 ml.) for 5 hr. The cooled solution was filtered, water (50 ml.) was added and the yellow precipitate which separated was collected (yield 0·1 g. approx.).

This was recrystallized five times from 40 % acetic acid and was obtained as golden brown rods (M.P. 248°). Found (dried at 140° *in vacuo*) C, 52·0; H, 2·9; MeO, 0·0 %. $C_{15}H_9O_5Br$ requires C, 51·6; H, 2·6; MeO, 0·0 %. A bromine estimation on a minute quantity of the compound indicated the presence of one Br atom.

20 mg. of the bromo-derivative were boiled in solution with 200 mg. of silver acetate in 50 ml. water and 10 ml. acetic acid. Some silver bromide was formed which was filtered from the hot solution. On cooling demethylated carviolin separated. It was recrystallized from 75 % acetic acid from which it separated in golden yellow rods. The amount was too small to recrystallize to constant melting point. M.P. 278–280°. Found (dried at 110° *in vacuo*) C, 63·5, H, 3·7 %. $C_{15}H_{10}O_6$ requires C, 63·0; H, 3·5 %

Oxidation of carviolin

Carviolin (100 mg.) was dissolved in conc. H_2SO_4 (20 ml.) and Pb_3O_4 (250 mg.) was added a few mg. at a time over 20 min., with constant shaking. The blood-red colour gradually changed to blue. The solution was poured on to ice, and the deep bluish purple solution thus obtained was reduced with SO_2. The colour changed to eosin pink and a reddish precipitate separated. This precipitate, when dissolved in alcohol and ether, showed strong absorption bands at wave-lengths

of 565, 548, 525 and 508 mμ. Bands at approximately the same wave-lengths were observed for 1:4:5:8-tetrahydroxyanthraquinone and cynodontin in the same solvents.

Comparison of carviolin and ω-hydroxyemodin

ω-Hydroxyemodin (kindly presented by Prof. Raistrick) was methylated in the usual way with dimethyl sulphate and alkali. The product had the same M.P. as carviolin trimethyl ether (186°) and no depression of M.P. was observed when the two compounds were mixed.

SUMMARY

Carviolin, a pigment of *P. carmino-violaceum* Biourge, has been shown to be a monomethyl ether of ω-hydroxyemodin. The compound has probably a free β-hydroxyl group.

I am indebted to Prof. J. M. Gulland and Prof. H. Raistrick for much helpful advice, and to Prof. Raistrick for samples of cynodontin and ω-hydroxyemodin.

REFERENCES

Graves & Adams (1923). *J. Amer. chem. Soc.* **45**, 2439.
Hind (1940). *Biochem. J.* **34**, 67.
Raistrick (1938). *Ergebn. Enzymforsch.* **7**, 333.

70. RELATION OF SKIN LESIONS IN THE RAT TO DEFICIENCY IN THE DIET OF DIFFERENT B_2-VITAMINS

By HARRIETTE CHICK, T. F. MACRAE and ALASTAIR N. WORDEN[1]

From the Division of Nutrition, Lister Institute, London, and Roebuck House, Cambridge

(*Received 1 March 1940*)

A noticeable feature about the earlier studies of the "dermatitis" occurring in rats when they are deprived of the heat-stable B-vitamins has been the irregularity of its incidence. The occurrence of two different types of skin lesions developing under these conditions was foreshadowed in earlier work [Chick & Roscoe, 1928, p. 795; Chick & Copping, 1930, p. 933] and has been more clearly demonstrated in recent investigations. As the members of the vitamin B_2 complex have gradually been separated from one another, and since vitamin B_1 has been available in the purified state, it has been possible to use basal diets supplemented with relatively pure preparations of one or other constituent [see György, 1934; Chick *et al.* 1935; Copping, 1936].

Type (*a*) is a florid dermatitis, roughly symmetrical, with swelling and redness of the digits of the paws and of the ears, "spreading over the limbs and trunk with cracking and desquamation of the skin", the so-called "rat pellagra" originally described by Goldberger & Lillie [1926] and called "rat acrodynia" by György [György *et al.* 1937]. In the present paper the expression "dermatitis" is reserved for this skin disease.

The lesions of type (*b*) occur more particularly in the skin over the head and chest with loss of fur and without swelling or inflammation. The fur becomes very scanty, especially over the head and forearms, and appears moist and matted, with bald patches of skin developing later. The eyelids and the nostrils become inflamed and the former are often stuck together with a serous, reddish fluid, which also exudes from the nostrils. The wrists become stained with this fluid from continual rubbing against the nose and eyes, and patches of fur round the vagina or penis also become stained by soiling with the urine.

With the discovery that riboflavin was a nutritionally active constituent of autoclaved yeast extracts and that the vitamin B_2 complex could be separated into riboflavin and a "supplementary substance", evidence was soon forthcoming that type (*a*) skin lesions developed on diets containing riboflavin and that riboflavin had curative value for skin lesions of type (*b*) [György, 1934; Copping, 1936].

Similarly, type (*a*) dermatitis yielded to treatment with the "supplementary factor" in the vitamin B_2 complex, which was called vitamin B_6 by György [1934]. This substance was found present in the "Peters's eluate" from charcoal as prepared from yeast extract. It was later found to be adsorbed from yeast on fuller's earth [Birch & György, 1936] and has since been separated in the pure crystalline state [Lepkovsky, 1938; György, 1938, 2; Kuhn & Wendt, 1938;

[1] Research Student in Animal Health, Ministry of Agriculture.

Keresztesy & Stevens, 1938; El Sadr *et al.* 1939, 2]. Its constitution has been investigated and its synthesis has also been accomplished [Harris & Folkers, 1939]. It has been called adermin but is usually known as vitamin B_6.

Chick *et al.* [1935] had also demonstrated the existence of a heat-stable substance which stimulated the growth of rats receiving vitamin B_1 and riboflavin, but this substance was present in the filtrate after adsorption of an autoclaved yeast extract with fuller's earth. At that time we assumed that this substance was identical with György's vitamin B_6 and we called it by that name [Chick *et al.* 1935; Copping, 1936]. This material, however, in contradistinction to György's, had no consistent curative effect on the florid dermatitis (type *a*); in fact in some cases the condition deteriorated after its administration [Copping, 1936].

Later investigations of the vitamin B_2 complex have demonstrated the existence of at least two heat-stable substances in yeast extract in addition to riboflavin, both of which are required to supplement vitamin B_1 in the diet of the rat [see Edgar & Macrae, 1937]. One, originally called the "yeast eluate" factor and now known to be identical with vitamin B_6 [El Sadr *et al.* 1939, 2] is adsorbed on fuller's earth together with riboflavin; the second, the "yeast filtrate factor" is in the filtrate. Similar fractions have been prepared by numerous workers from liver, yeast and rice polishings. Separation has been achieved by means of fuller's earth in almost all these cases, the one factor (vitamin B_6) being adsorbed and subsequently eluted and the second factor being found in the filtrate.

It is obvious, therefore, that the "supplementary substance" investigated by Chick *et al.* [1935] and by Copping [1936] contained not György's vitamin B_6, as was supposed, but the filtrate factor. Its failure to act upon rat dermatitis was therefore not inconsistent with the observations of György on "vitamin B_6".

It was later found that vitamin B_6 is present in relatively high concentrations in cereals and cereal products [Birch *et al.* 1935]. Copping [1936] found that rat dermatitis, which was untouched by her "supplementary factor" from yeast, was swiftly cured by an alcoholic extract of whole maize or wheat. These facts may be important in explaining the irregular incidence of dermatitis in the earlier work on this subject, since the basal diets almost invariably contained starch of cereal origin, which may well have been contaminated with vitamin B_6 (see also below, p. 591). Substitution of starch by sugar in the diet was found to favour the occurrence of dermatitis [Chick *et al.* 1935]. With a diet containing sugar as source of carbohydrate and with the purified "Peters's eluate" previously given as source of vitamin B_1 replaced by pure crystalline aneurin, as in the experiments recorded below, rat dermatitis is almost invariably developed in the absence of the yeast eluate factor (vitamin B_6).

It should be noted that more recent work in this laboratory [El Sadr *et al.* 1939, 1], carried out since the present experiments were arranged and after most of them were completed, has shown that the "yeast filtrate factor" is itself complex and contains at least two at present unidentified nutrients essential for the rat: one, extractable with amyl alcohol, is the factor studied in this paper and called the "purified filtrate factor", the other is non-extractable with amyl alcohol. It has been shown further that at least one vitamin belonging to the vitamin B_2 group is contained in whole liver in addition to riboflavin and the nutrients present in the yeast eluate fraction (vitamin B_6) and in the yeast filtrate fractions. These facts must be remembered in drawing conclusions from the results of the present work, because there is the possibility that other deficiencies, in addition to those recognized, might have complicated the results observed.

The present experiments fall into two series. In the first, young newly weaned rats received a basal diet containing pure vitamin B_1 and at most one of the members of the vitamin B_2 complex; the symptoms developed were observed, and the effect on them of adding certain of the missing B_2-vitamins one by one. In the second series the effect was observed of feeding diets containing at least two of the B_2-vitamins, and of the result of restoration of certain of the missing factors.

Experimental

Series I. Methods used

The first series of observations was made on 32 young rats from 4 litters, of body weight ranging from 36 to 53 g. at weaning. The rats received the usual "W.G.L.-B" cooked diet[1] of this laboratory containing rice starch as carbohydrate and with 5μg. pure vitamin B_1 (aneurin chloride) hydrochloride and 0·08 g. cod liver oil given daily to each animal separately. After 3–4 days, the basal diet was changed to the following: purified casein (Glaxo ashless extracted) 22, sucrose 67, lard 5·5, salt mixture (McCollum's No. 185) 5·5 parts. Lard was given to ensure an adequate supply of the unsaturated fatty acid dietary factor, as it was obvious that the effect on the skin of any deficiency of this nutrient essential would seriously complicate the study of skin lesions which might be due to other defects in the diet. The food was kept as a dry powder and after weighing was mixed with water to the consistency of a thick cream in order to prevent loss by scattering.

The rats were divided into 4 groups, of which the members were distributed as evenly as possible over the 4 different litters. Group 1 (8 rats) received the basal diet with aneurin and cod liver oil only; group 2 (10 rats) received in addition 12μg. daily of pure riboflavin; group 3 (8 rats) received the purified yeast filtrate factor prepared by extraction with amyl alcohol, in a daily dose equivalent to 1 g. yeast (dry wt.); group 4 (6 rats) received the yeast eluate fraction (vitamin B_6) in amount equivalent to 2 g. yeast (dry wt.) daily.

The preparation of the purified filtrate factor from yeast extract was made by the method described by Edgar *et al.* [1938]. The eluate fraction was prepared from the fuller's earth adsorbate from yeast extract as described by Edgar & Macrae [1937]. In certain cases more highly purified preparations of the eluate fraction were employed.

The rats in the different groups were kept without any further addition to the diet for several weeks or until definite symptoms were observed. Careful watch was kept for the first occurrence of the dermatitis (type *a*) and also for the appearance of conjunctivitis and staining of the fur (type *b*). After the occurrence of dermatitis was noticed, addition was made of one of the missing constituents of the vitamin B_2 complex and the results noted, the other constituent or constituents of the complex being added later. As all the rats were deprived of two or more members of the vitamin B_2 complex growth was very poor, as might be expected. On the whole, those in group 4 receiving the eluate fraction remained in the best condition, at least for several weeks.

Results

No dermatitis occurred in the 6 rats in group 4 receiving the "yeast eluate" fraction but, with one or two exceptions mentioned below, it appeared regularly in the other 3 groups after periods varying from 6 to 13 weeks on the experimental diets.

[1] "Glaxo ashless extracted" casein 100, rice starch 300, cottonseed oil 60, lard 15, salt mixture (McCollum's No. 185) 25, water 500 parts; cooked by steaming for 3 hr.

Table 1. *Incidence of dermatitis in young rats maintained on basal diet with daily doses of 5 μg. aneurin and 0·08 g. cod liver oil together with the other supplement indicated*

Group	Supplement daily dose	No. of rats	No. developing (type *a*) dermatitis	Time on the diet before onset, weeks	No. showing (type *b*) skin lesions, conjunctivitis, exudation from eyes and nostrils	Time on the diet before onset, weeks
1	0	8	6	6–12	4	3– 9
2	Riboflavin 8–17μg.	10	9	6–12	7	5–12
3	Filtrate fraction ≡1 g. yeast (dry wt.)	8	7	8–13	4	6–10
4	Eluate fraction (vitamin B_6) ≡2 g. yeast (dry wt.)	6	0	—	5	2–12

Only one rat (No. 4) in the group deprived of eluate fraction and receiving filtrate fraction failed to develop dermatitis in 10 weeks, but later this animal developed it after riboflavin was given. Two rats (Nos. 17 and 18) in group 1, receiving no supplement, showed no signs after 10–11 weeks, but of these one developed the symptoms also after receiving riboflavin for 9 days. In the riboflavin group, all the rats with the exception of one, No. 29, showed the usual symptoms. The digits of the fore paws were usually the first to be affected, becoming roughened, then red, swollen and inflamed, the inflammation spreading in time to the whole paw, the same sequence of events taking place later in the hind paws. The skin at the tip of the nose and at the corners of the mouth was later attacked, and also the tips of the ears. Often the characteristic thickening of the ears appeared only some weeks after the paws were affected and sometimes only after addition to the diet of riboflavin or the filtrate factor (see below, p. 584). Whether this fact was connected with these additions to the diet or depended only on the further lapse of time cannot be decided.

The (*b*) type of skin lesions with conjunctivitis and serous secretion from eyes, nostrils etc. and matted fur was developed in some animals in all the groups, usually earlier than the dermatitis, if this occurred. It was particularly severe in some of the rats in group 4 receiving the eluate fraction, but deprived of riboflavin and filtrate factor, and in these cases was uncomplicated by simultaneous presence of dermatitis. Sometimes the whole fur was stained red, but the deepest stains were on the wrists, round the nostrils and eyes, and on the lower lip, as well as round the penis or vagina and on the tail. The red pigment could be removed with cotton wool moistened with water. The pink coloured solution gave none of the reactions for haemoglobin or blood pigments, the benzidine test being wholly negative. The red substance excreted was found by Mrs C. E. Work in this department to give the absorption bands of a porphyrin and was identified with protoporphyrin. Excretion of a porphyrin in the urine of rats deprived of B_2-vitamins was reported by Ellinger *et al.* [1935]; this was considered to be probably coproporphyrin.

Curative tests

i. *Effect of eluate fraction on dermatitis.* The action of the eluate fraction in preventing (type *a*) dermatitis was obvious in group 4 where none of the 6 rats which received it showed any symptoms. To test its curative action, 7 rats in groups 1, 2 and 3 with marked dermatitis received the various preparations of eluate fraction described above in daily amounts equivalent to 2 g. dry yeast.

Of the 4 rats which previously received riboflavin, good cures of the characteristic dermatitis occurred in about 3 weeks in 3 (Nos. 13, 14 and 22) after administration of the more highly purified preparations of the eluate fraction and a slower cure, taking $4\frac{1}{2}$ weeks, was observed in 1 (No. 21). Of 2 cases of dermatitis in group 3 receiving filtrate fraction, one (No. 11) was cured in 3 weeks with a highly purified eluate fraction; in the case of the other (No. 19) the cure was noted as "incomplete" after 2 weeks, and the fur grew better and became more normal after riboflavin was also given. One of the rats (No. 10) developing (type *a*) dermatitis in group 1, in which no supplement had been given, showed improvement with eluate fraction but died before this had progressed far.

ii. *Effect of riboflavin on (type* a) *dermatitis.* The effect upon dermatitis of giving riboflavin to rats previously deprived of it was tried in 9 cases. Growth was always stimulated by this addition and frequently there was a temporary remission in the swelling and inflammation of the paws, but the thickening and inflammation of the rims of the ears was never checked, and frequently developed after riboflavin had been given.

Of 3 rats with dermatitis in group 1 (no addition), treated with riboflavin, one (No. 9) became worse, but 2 (Nos. 1 and 3) showed temporary marked improvement of the swollen paws. In one of these (No. 3) there was a subsequent relapse and in the other (No. 1) the cure remained incomplete; in both cases the ears became inflamed only after riboflavin was given and subsequent addition of eluate fraction improved matters very greatly. Another rat in this group (No. 17) which had not developed dermatitis in 11 weeks showed symptoms 9 days after receiving riboflavin.

Of 4 rats with dermatitis in group 3 (receiving filtrate fraction) the symptoms improved after receiving riboflavin in 2 cases; one of these (No. 27), however, died shortly afterwards and the other (No. 12) relapsed subsequently. The remaining 2 rats (Nos. 2 and 20) showed no improvement when riboflavin was given, but these, as well as No. 12, were cured when eluate fraction was added. In the case of No. 20, a rapid cure was made with pure crystalline vitamin B_6 (20μg. daily). In addition to the above, one rat (No. 4) in this group, which showed no symptoms after 11 weeks, developed them in 2–3 weeks after riboflavin was given. With 4 of the above 5 rats it was noted that the ears showed the characteristic thickening, inflammation and hardening of the edges only after riboflavin had been given.

One rat (No. 30) developed dermatitis after 6 weeks on the basal diet with riboflavin but the skin trouble steadily improved during the subsequent 5 weeks; it then relapsed but again showed remission of the dermatitis. However, the fur remained very unhealthy and the animal finally died.

iii. *Effect of filtrate fraction upon dermatitis.* The effect of adding filtrate fraction to the diet of rats showing (type *a*) dermatitis was tried in 5 cases. In rat 26 (no supplement) the dermatitis of the paws became worse and the ears became affected. With rats 5 and 8 (receiving riboflavin) the ears also developed dermatitis after the filtrate fraction was given, but the inflammation of the paws subsided; there was, however, a subsequent relapse and final cure only after the eluate fraction was also given. In the riboflavin group rat 7 showed some improvement when receiving filtrate fraction but died, and rat 29 deteriorated after filtrate fraction was given, but improved when the eluate fraction was also added to the diet.

Occurrence of (type b) *skin symptoms, with sticky exudation from conjunctiva and nostrils, staining of fur and later occurrence of bald patches*

This condition, described in detail above (p. 580) as type *b*, was seen to a slight degree in 7 and in more marked degree in 20, out of the 32 rats. Of the latter 20, 13 belonged to the group which had been deprived of riboflavin, and 7 to the group which had received it from the beginning of the experiment. This result did not agree with our previous conclusion that this type of skin abnormality was produced in rats when riboflavin was absent from the diet and was cured promptly when it was given (see above, p. 580). In 8 of the above 13 cases deprived of riboflavin, the symptoms disappeared or showed improvement when riboflavin was given. In the other 5 cases they persisted to some degree in spite of administration of riboflavin and disappeared only when other B_2-vitamins were also added to the diet.

The results detailed in the preceding paragraphs may be summarized thus. In the 6 rats which received eluate fraction from the beginning of the experiment no (type *a*) dermatitis occurred, but it was noted in 25 of the 26 rats deprived of this fraction in periods varying from 6 to 12 weeks. Of these 25 rats, 7 were treated at once with preparations of the eluate fraction, and in all cases cures were observed, though the fur remained stringy and poor with bald patches of skin, and the rats did not usually recover a normal coat until the other missing constituents of the vitamin B_2 complex were supplied.

Of the 14 rats with (type *a*) dermatitis, which were treated with riboflavin or filtrate factor or both, 8 showed an improvement or a temporary cure, especially of the skin lesions of the paws, but relapses occurred. Frequently the skin lesions were developed on the ears only after riboflavin or filtrate fraction had been given. In the above 8 and in the remaining 6 rats, cures took place later when the eluate fraction was supplied. In two cases this was given as pure crystalline vitamin B_6 kindly supplied by Dr Lepkovsky.

While the (type *a*) dermatitis in rats described above on p. 580 appears to be a specific result of deprivation of vitamin B_6 (eluate fraction), the development of the eczematous skin disease showing the (*b*) type of symptoms is evidently not to be similarly related to deprivation of riboflavin. The above results indicated that the maintenance of a healthy skin in rats depends on an adequate provision of all B_2-vitamins, a conclusion confirmed by the results of the second series of experiments described below.

Series II. Methods used

The general method used in the previous series of experiments is one that is open to criticism, in that the symptoms observed will probably have been the result of the multiple deficiency which was always present—seeing that in the earlier periods of the tests only one of the vitamin B_2 fractions was provided. It was therefore decided to observe over long periods of time the effects of diets arranged to provide the rats with at least two members of the vitamin B_2 complex in optimal amount and study the results of deprivation of one.

Accordingly, groups of young rats were observed which, from the time of weaning, received

(1) the yeast eluate fraction (vitamin B_6) and the purified yeast filtrate fraction, but no riboflavin;

(2) riboflavin and yeast eluate fraction, but no yeast filtrate fraction;

(3) riboflavin and purified filtrate fraction, but no yeast eluate fraction.

The rats, like those in Series I, were given the "W.G.L.-B" diet for 3–5 days from weaning, supplemented with pure vitamin B_1 and cod liver oil. Thereafter they received a diet constituted somewhat differently from that given in the first series. It contained purified[1] casein 22, sucrose 66, wheat germ oil 2·2, linseed oil 3·3, salt mixture (McCollum's No. 185) 5·5, parts. In this ration the lard of the previous diet was replaced by the mixture of linseed oil and wheat germ oil, the former to provide the essential unsaturated fatty acids which were previously supplied in the lard, and the latter to supply vitamin E which, regarding the long duration of the experiments, it was thought wise to include.

The following supplements were given separately to each rat daily: cod liver oil 0·08–0·16 g.; pure aneurin chloride hydrochloride (vitamin B_1) 5–20 μg.; riboflavin 16–28 μg.; purified filtrate fraction in amount corresponding to 1–3 g. yeast (dry wt.); eluate fraction in amount corresponding to 2 g. yeast (dry wt.), or crystalline vitamin B_6 10–15 μg. The amounts given varied with the body weight of the rats.

The carbohydrate in the diet was varied and the effect of the variation studied. In some instances the sucrose was replaced by an equal weight of purified starch, either rice starch or maize starch. The maize starch was the purest obtainable and contained 0·03–0·04 % nitrogen. The ingestion of raw starch introduced the danger of refection and this complication occurred in 3 instances. In 2 cases (rats 33 and 52) it was cured by moving the rats to cages with wire floors of very wide mesh and thus hindering coprophagy; in one case the rat (No. 66) had to be discarded. The effect of cooking the starch-containing diet, as in the preparation of our usual "W.G.L.-B" diet (see above, p. 582), was also investigated. It has been frequently observed in this laboratory that if the cooked starch diet were replaced by one containing sucrose, there was a general deterioration in the condition of rats deprived of eluate fraction. If, however, the starch-containing diet was fed in the raw state there was much less difference. The results obtained in Exps. 3 and 4 below confirm this result and suggest that starches of cereal origin, even when purified, may be contaminated with traces of vitamin B_6 which are made more available after they have been cooked with water.

Exp. 1. 16 rats from 2 litters were divided at weaning evenly over 3 groups as follows: 2 groups of 4 rats were deprived respectively of riboflavin and of filtrate factor; these received the diet containing sucrose as carbohydrate. A group of 8 rats was deprived of eluate fraction (vitamin B_6); of these 4 received the sucrose diet, 2 the rice starch and 2 the maize starch diet. All the diets were given raw and were mixed with a little water to form a thick paste before being fed. The results are summarized in Table 2 and growth curves given in Fig. 1.

Riboflavin deprivation. Exp. 1 (a)

The group deprived of riboflavin made by far the poorest progress. There was no significant increase in weight; one rat (No. 41) of initial weight 42 g. which survived 20 weeks weighed only 54 g. at the end of the period (see Fig 1*a*). The skin was badly affected, showing the type (*b*) symptoms described above; there was erosion round the eyes which were sunken; the lids were stuck together with the serous exudate and there was dullness of the cornea; the fur was matted and eczematous, the skin in some places was exfoliating and in others nearly bare of fur, the wrists were badly stained with a reddish exudate. Two of the 4 rats,

[1] "Glaxo" extracted, ashless casein, further purified by washing 6 times with 2% NaCl solution followed by dilute alcohol and drying in a current of dry air.

Table 2

Exp. no.	Litter no.	Deficiency in diet	Nature of carbohydrate in diet	No. of rats	Av. body-weight* (g.) after weeks on diet: Initial	8	16	24	32	Incidence of type (*a*) dermatitis: No. of cases	Time of onset, weeks	Incidence of type (*b*) skin lesions: No. of cases	Time of onset, weeks	Remarks
1 (*a*)	5/6	Riboflavin	Sucrose	4	42	48	57 (1)	—	—	—	—	4	4–8	Eye lesions specially noticed in group (*a*); reddish staining of coat and greying of pigmented fur in group (*b*)
(*b*)	5/6	Filtrate fraction	,,	4	44	78	108 (3)	114 (3)	116 (3)	—	—	4	5–11	
(*c*)	5/6	Eluate fraction (vitamin B_6)	,,	4	42	66	88 (1)	88 (1)	86 (1)	3	8–9	—	—	1 rat cured with vitamin B_6 at 10 weeks
		,,	Rice starch, raw	2	43	74	77 (1)	—	—	2 (slight)	12–20	—	—	
		,,	Maize starch, raw	2	41	81	91	88 (1)	92 (1)	—	—	—	—	
2	7	,,	Sucrose	2	54	88	111	—	—	2	10–11	—	—	
			Rice starch, raw	3	52	94	96	103 (2)	—	2	10–24	—	—	1 rat with dermatitis died at 17 weeks. 1 was cured with vitamin B_6 at 31 weeks
			Maize starch, raw	2	54	88	100	110	—	2	10–25	—	—	
3	8/9	Eluate fraction (vitamin B_6)	Sucrose	6	45	78	99 (5)	101 (3)	111 (2)	5	8–16	—	—	1 rat with severe dermatitis cured with vitamin B_6 at 12 weeks. 1 rat died at 19 weeks
			Rice starch, cooked	5	45	139	160	158	164	—	—	—	—	Observed for 35 weeks
			Rice starch, raw	4	44	81	102 (3)	94 (2)	116 (1)	3	8–24	—	—	Dermatitis only slight, 1 rat remained free for 35 weeks
4 (*a*)	10	Eluate fraction (vitamin B_6)	Rice starch, raw	2♂	50	101	108 (1)	122 (1)	123 (1)	4	8–30	—	—	Dermatitis slight, chiefly ears
				2♀	47	86	91	101	105					
(*b*)	10	Addition of vitamin B_6 10–15 μg. daily	,,	2♂	50	172	221	262	291	—	—	—	—	All completely healthy after 34 weeks
				2♀	47	160	184	206	212					

* Nos. in brackets signify nos. of animals from which average is calculated.

after 8 and 10 weeks respectively, were killed to provide post-mortem material for histological study by Dr El Sadr of this department. The 4th rat (No. 44) after 9 weeks on the diet weighed only 45 g. and then received 11 μg. riboflavin daily. Although its condition was very bad, it at once began to grow, gaining 15 g. in the following 9 days and there was an immediate improvement in the skin condition. The eyelids which had been stuck together with the serous exudate became wide open and the opaque condition of the cornea vanished, the coat

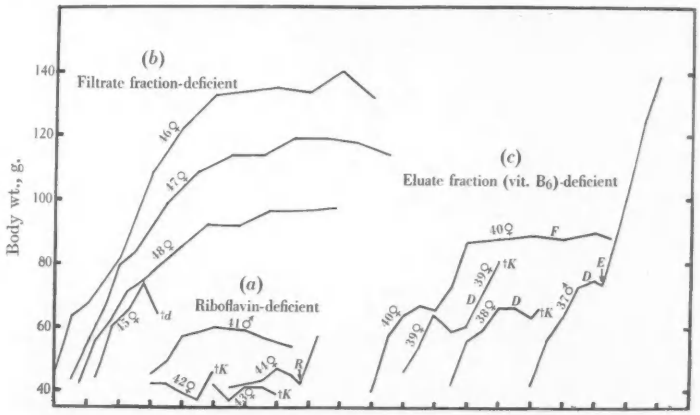


Fig. 1 (see Table 2, Exp. 1 *a*, *b*, *c*). Growth curves of litter-mate rats on diet containing sucrose as carbohydrate, with supplements of cod liver oil, pure vitamin B_1 and the following B_2-vitamins: (*a*) yeast eluate fraction and purified yeast filtrate fraction; (*b*) riboflavin and yeast eluate fraction; (*c*) riboflavin and purified yeast filtrate fraction. *D* signifies onset of dermatitis. *F* signifies onset of fits. *R* signifies administration of riboflavin. *E* signifies administration of eluate fraction (vitamin B_6). †*d* signifies died; †*K* signifies killed, very ill. The divisions on the abscissa represent periods of 4 weeks.

was much improved and the staining of the fur and the abdomen almost disappeared. The condition of these rats deprived of riboflavin was as described elsewhere by El Sadr [1940]. Cataract was not observed, but only in the case of one rat was the duration of the deprivation sufficient for this to occur.

Filtrate fraction deprivation. Exp. 1(b)

The 4 rats deprived of filtrate factor remained in much better condition than those deprived of riboflavin. Three slowly attained a body weight of 100 g. and over during the first 16 weeks on the diet but, after this, little more increase occurred and 2 female rats which survived 12 months weighed only 127 and 126 g. respectively. The eyes were not affected, symptoms being confined to the skin; the fur became matted and stringy and stained with the reddish secretion containing protoporphyrin mentioned above (p. 583). The stain is soluble in water and the animals could be cleaned by washing and frequently they cleaned themselves, but in the course of a few days became stained again. Two of the 4 rats showed greying of their dark pigmented fur, especially over the head and shoulders. Although stained fur was noted in some cases of rats deprived of riboflavin when in a very bad state, and also in those deprived of eluate fraction (vitamin B_6) for long periods, this reddish staining of the fur, especially of the

abdomen in the earlier stages of deprivation, seems to be characteristic of filtrate fraction deficiency, as the eye lesions described above are specific for riboflavin deficiency. The 2 rats which survived a year without filtrate fraction became nervous and excitable but, in contrast to those deprived of eluate fraction, readily took their daily doses and no fits were observed (see below).

The rats in this series deprived of riboflavin and filtrate factor were few in number, but the symptoms observed were confirmed by more numerous observations by other workers in this department.

Deprivation of eluate fraction (vitamin B_6). Exps. 1(c), *2, 3, 4, Table 2*

Of the 4 rats in Exp. 1 receiving sucrose as carbohydrate, 3 developed type (*a*) dermatitis in 8–9 weeks. The 2 rats receiving raw rice starch as carbohydrate behaved similarly but the dermatitis was less severe; the 2 rats receiving maize starch had shown no dermatitis after 13 and 33 weeks' observation respectively.

In Exp. 2 dermatitis occurred in the rats receiving maize starch in their diet and in 2 of the 3 rats having rice starch but later than in the 2 litter-mates receiving sucrose (see Table 2). It was noted that with these rats developing dermatitis and the others doing so in Exps. 3 and 4 the sequence in which the different sites of the skin showed inflammation was different from that observed in the experiments of Series I. In the experiments of Series II, the skin of the edges of the ears and that on the nose was usually the first to be affected, and the paws became inflamed later. Sometimes the paws remained without inflammation for a very long period; this was frequently the case when the diet contained starch as carbohydrate. The rats in Series I, deprived of riboflavin or filtrate factor as well as of vitamin B_6, invariably showed the earliest lesions in the paws, the ears becoming affected, with thickening and inflammation of the edges, much later and frequently only after riboflavin had been added to the diet (see Plate 2, fig. 5).

When pure vitamin B_6, 15μg. daily, was given to the rats with these skin symptoms, swift cures took place. Similarly, those rats which, in addition to aneurin, riboflavin and purified filtrate factor, received also a supplement of pure vitamin B_6, 10–15μg. daily, from the beginning of the experiment, developed no dermatitis and grew into fine, apparently normal rats, the males reaching over 300 g. and the females over 200 g. weight in about 7 months (see Table 2, Exp. 4 (*b*)). The males and females were mated and normal pregnancies occurred, two healthy litters of 9 and 12 live young were born but the mothers were unable to lactate and the young died within 48 hr. The growth curves of these rats and of their deprived litter-mates are shown in Fig. 3 and a photograph is given in Plate 2, fig. 4.

After long periods of deprivation, 20–30 weeks and often sooner on the sucrose diet, changes other than those of (type *a*) dermatitis were noted in the skin and fur of rats deprived of vitamin B_6. The fur became scanty and matted, with bad areas especially on the chin and chest, over the forepaws and around the eyes. The spectacled state of the eyes was sometimes so marked as to resemble that resulting from deprivation of riboflavin. There was staining of the fur with the reddish pigment, found by Mrs C. E. Work to have the absorption bands of protoporphyrin; the colour of the fur was also affected, the white parts becoming yellowish and the pigmented parts rusty in colour, especially on the abdomen and chest.

The rats which survived deprivation of vitamin B_6 for long periods, usually not less than 4–5 months, also developed a tendency to fits. These formed the subject of special study and are dealt with in a separate paper [Chick *et al.* 1940].

Influence of carbohydrate in the diet

The 5 animals in Exp. 3 receiving the basal diet containing cooked starch had no dermatitis; they showed better growth and remained in much better general

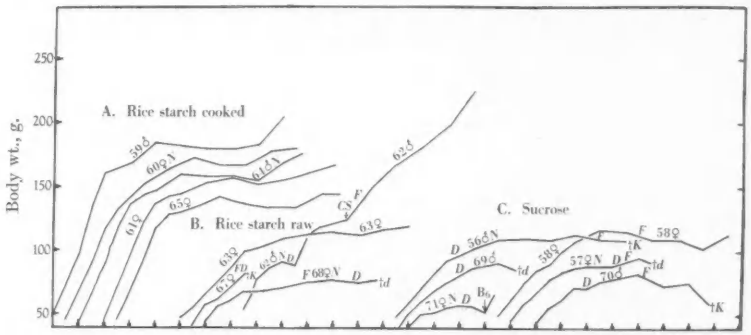


Fig. 2. Growth curves of litter-mate rats on synthetic diet deprived of eluate fraction and containing carbohydrate. A, as rice starch (cooked); B, as rice starch (raw); C, as sucrose; *N* signifies supplement of nicotinic acid, 2 mg. daily; *D* signifies onset of dermatitis; *F* signifies onset of fits; †*K* = killed, †*d* = died; at *CS* rat 62 received the cooked diet; at *B* rat 71 received pure vitamin B_6. The divisions on the abscissa represent periods of 4 weeks. (Table 2, Exp. 3.)

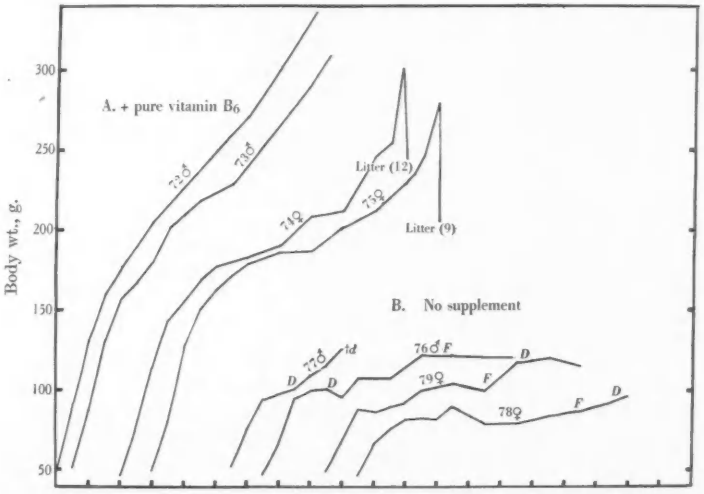


Fig. 3. Growth curves of litter-mate rats on synthetic diet (raw) containing rice starch as carbohydrate and deprived of eluate fraction (vitamin B_6). Group A received pure crystalline vitamin B_6, 10–15 μg. according to size. Group B received no supplement. *D* signifies onset of dermatitis; *F* signifies onset of fits; †*d* signifies died. The divisions on the abscissa represent periods of 4 weeks. (Table 2, Exp. 4 and Plate 2, fig. 4.)

condition than their 4 litter-mates receiving the same basal diet fed in the raw state (see Table 2 and Fig. 2). The latter behaved similarly to the groups having the

sucrose diet, except that the dermatitis was definitely less severe and usually took longer to develop. One explanation of this difference would be that the rats on the starch diet were receiving a small but inadequate amount of vitamin B_6. This vitamin is known to be present in cereals in fair amount (see above, p. 581) and has the property of being readily adsorbed on suitable materials. It is possible therefore that some small amount might remain attached to starches of cereal origin, even after purification by washing. Tange & Kaneko [1938] reported that vitamin B_6 was unnecessary in the diet of the rat when maize starch was given as carbohydrate and György *et al.* [1937] found that "acrodynia" was developed regularly in 6–20 weeks in rats deprived of vitamin B_6 when the rice starch in the diet was replaced by sucrose. Oleson *et al.* [1939, 1] observed better growth in rats on synthetic diets, apparently adequately supplied with B-vitamins, when the sucrose of the basal diet was replaced by dextrin.

The vitamin B_6 present in the raw rice starch used in the present work appeared, however, to be combined in some form in which it is relatively unavailable for the rat, but from which it could be freed and rendered more digestible by cooking with water. Even so, the amount appears to be small, for the rats in Exp. 3 receiving the cooked starch diet, although they developed no dermatitis, were much inferior to those in Exp. 4 which received an adequate daily dose of pure vitamin B_6 (see Figs. 2 and 3).

This effect of cooking has so far been investigated only with rice starch. Raw maize starch, however, also showed some protective power against the effect of vitamin B_6 deprivation (see Table 2, Exps. 1 (*c*) and 2) and experiments are now in progress to discover if this power is increased when the starch is cooked.

Influence of nicotinic acid

Of the 15 rats in Exp. 3, 2 of 5 receiving the cooked starch basal diet, 2 of 4 receiving raw starch and 3 of 6 receiving sucrose, were given 2 mg. nicotinic acid daily in addition to the supplements of aneurin, riboflavin and filtrate factor. In no way did these rats differ in behaviour from their litter-mates which received no supplement of nicotinic acid. There was no other source of nicotinic acid in the diet of these rats, seeing that all were deprived of the "yeast eluate fraction" which has sometimes been found to contain nicotinamide [Macrae & Edgar, 1937]. Fresh evidence is thus given for the conclusion that nicotinic acid is not required for the nutrition of the rat [see Chick *et al.* 1938; Macrae & Edgar, 1937; Cook *et al.* 1937].

As was pointed out above (p. 581), some of the diets used in the experiments of Series II were deficient in certain, at present unknown, members of the vitamin B_2 complex, in addition to the deficiency which was recognized. Traces of these factors may, on the other hand, have been provided in the purified casein, or in the purified starch, and have been absent when the carbohydrate was supplied as sucrose. When the basal diet contained starch, a partial protective action was certainly exercised against the effects of vitamin B_6 deficiency, and when it was adequately supplemented with aneurin, riboflavin, purified filtrate factor and vitamin B_6, the rats developed well, remained healthy and even reproduced (Table 2, Exp. 4*b*).

With the reservations contained in the preceding paragraph it may be concluded: (1) that the nutritional dermatitis in rats showing the type (*a*) characteristics, formerly called "rat pellagra" and "rat acrodynia" by some authors, is the result of deprivation of vitamin B_6; (2) that an eczematous skin disease affecting chiefly eyes and nose is caused by riboflavin deficiency; (3) that a

similar affection, not including the eyes, but with greying of pigmented fur and reddish staining of the fur and abdomen, occurs with deficiency of filtrate factor. But loss of fur round the eyes with exudation of a reddish secretion, an unhealthy condition of the skin with matted stringy fur, often causing bald patches of skin, especially on the neck and chest and forearms, was seen with deprivation of each of the 3 above members of the vitamin B_2 complex, especially when the period of deprivation was prolonged.

The existence of a specific relation between vitamin B_6 and type (*a*) dermatitis or "rat acrodynia" has been confirmed by numerous workers in this field since it was first observed by György [1934]. As regards the other members of the vitamin B_2 complex, it is agreed that each is required for satisfactory growth, but there is at present little agreement as to the pathological effects of deprivation of the separate vitamins.

György *et al.* [1937] described a "haemorrhagic" condition with nose bleeding, bloody secretion from the eyes and haemorrhage in the adrenals, accompanied by anaemia of an aplastic type, in rats maintained on a synthetic diet containing sucrose as carbohydrate, with supplements of vitamin B_1 and riboflavin. The rats also developed "acrodynia", which was cured by pure vitamin B_6; the haemorrhagic condition, on the other hand, remained unchanged but yielded to crude preparations of vitamin B_6. In a later paper György [1938, 1] concluded that the anaemia-preventing substance was nicotinic acid. This conclusion is not confirmed by the present study and it must be noted that the filtrate factor was absent from György's diet. In a more recent communication György & Eckhardt [1939] describe three different types of skin disease of an eczematous nature with loss of hair and sticky secretion from the eyes, sore nose and mouth and scaliness of the skin, developed in rats maintained for long periods on a synthetic diet supplemented with purified vitamin B_1, pure vitamin B_6 and riboflavin. All these affections yielded to administration of the filtrates from wheat germ, yeast and rice polishings after treatment with fuller's earth.

In a recent paper Oleson *et al.* [1939, 1] have stated that, in addition to "acrodynia" which could be cured by vitamin B_6, three separate syndromes are developed in rats receiving aneurin, riboflavin and nicotinic acid: (1) paralysis, (2) a haemorrhagic condition, (3) a condition of "eye erosion". The "eye erosion" was cured by a "filtrate fraction" prepared from liver extract after adsorption in acid solution with fuller's earth; the same fraction was later [Oleson *et al.* 1939, 2] found preventive of "achromotrichia", presumably greying of the pigmented portions of the fur. The latter conclusion is confirmed by us, the former not, since in our experiments rats deprived of filtrate factor showed no marked changes in the skin or fur round the eyes. Greying of the fur in rats deprived of filtrate factor has also been reported by Morgan *et al.* [1938] and by Lunde & Kringstad [1939]. The haemorrhagic condition described by Oleson *et al.* [1939, 1] resembled that studied by György *et al.* [1937]. Both it and the paralysis were cured by the eluate fraction but not by pure vitamin B_6. We have not observed any paralysis in rats deprived of our yeast eluate fraction for periods up to a year. On the other hand we have found a tendency to fits invariably developed after deprivation for 4–5 months (see the following paper).

Such discrepancies between the conclusions of different workers are perhaps inevitable and may be explained by differences in the basal diets used and in the degrees of purity of the vitamin supplements and may only be resolved when all the latter are available in the pure state.

At the same time it is more than probable that many pathological affections, including these of the skin, occurring in animals suffering from faulty nutrition

are not related to any specific deprivation. It is generally agreed that the skin and mucous membrane of an animal are particularly sensitive to nutritional defects. The skin covering the external surface of the animal and the mucous membranes lining the alimentary tract and nasal passages and covering the eyes, in one respect exercise similar functions, viz. the protection of the organism from invasion by external bacteria, which are abundantly present on the "exterior" surfaces of these membranes. When the animal is deprived of nutritional essentials, the defensive mechanisms of these tissues are disturbed, and invasion by bacteria causes a diseased condition. The points of union of skin and mucous membrane appear to be sites of particular vulnerability; such are the edges of the lips and nostrils and the corners of the eyes and the margins of the skin round the vagina. The common occurrence of furunculosis, conjunctivitis and angular stomatitis in ill nourished human beings may be reactions to general, rather than to specific, dietary deficiencies. Angular stomatitis is frequent among poorly fed native races, especially among children, and although foods containing the vitamin B_2 complex have been found curative, no single member of the group has yet been found effective when administered in the purified state.

Summary

1. Rats receiving purified synthetic diets with the addition of pure aneurin (vitamin B_1) and deprived of one or more of the B_2-vitamins were observed over long periods up to 1 year from weaning stage.

2. Rats deprived of riboflavin showed no significant increase in weight and developed an eczematous condition of the skin affecting specially nostrils and eyes; the rims of the latter became denuded of hair; there was dullness of the cornea, blepharitis and conjunctivitis, the eyelids being stuck together with a serous exudate. These symptoms cleared up rapidly when riboflavin was given.

3. Rats deprived of filtrate fraction showed slow subnormal growth up to a maximum weight of about 100–130 g. weight; they developed poor coats with matted and stringy fur which often became grey in the pigmented areas over the head and shoulders and was frequently stained with a reddish exudate containing protoporphyrin on the forearms and abdomen.

4. Rats deprived of eluate fraction (vitamin B_6) developed characteristic dermatitis, which was cured promptly when pure vitamin B_6 was given. After prolonged deprivation the rats became hypersensitive and developed a tendency to fits of an epileptic nature.

5. The relation of the skin symptoms described under 2, 3 and 4 to the known missing vitamin was not, however, always strictly specific. Dermatitis in rats deprived of vitamin B_6 and riboflavin showed temporary improvement when riboflavin was given. In prolonged deprivation of vitamin B_6 there occurred erosion of the fur round the eyes and frequently patches of eczematous skin with scanty matted fur and bald patches, resembling those occurring in deficiencies of shorter duration of riboflavin or of filtrate fraction. These facts may be explained by interference with the normal mutual interaction of the known members of the vitamin B_2 complex, or by absence from the diets of other unknown vitamins belonging to this group.

6. Starches, given as source of carbohydrate in the basal diet, exerted a protective action, as compared with sucrose, on animals deprived of vitamin B_6; this was greater when the diet was cooked. It is suggested that starches of cereal origin, even when purified, may retain traces of vitamin B_6 which are more readily available to the rat after the starch has been cooked with water.

In conclusion our thanks are due to other workers in this Department, including Dr L. Gravel of the Laval University of Quebec and especially Mr G. Flynn, for assistance in the care of the experimental animals. We are also indebted to the late Mr Walter Acton of Messrs Mackean, Paisley, for kindly arranging for a supply of pure maize starch and for information regarding its analysis; to Dr S. Lepkovsky, Berkeley, U.S.A., for a gift of pure vitamin B_6; to Dr S. Smith of Messrs Burroughs Wellcome & Co., for pure riboflavin, and to the firm of Roche Products, Ltd., for kindly providing pure aneurin (vitamin B_1) and riboflavin. The last stages of the experimental work were carried out at Roebuck House, Chesterton, Cambridge, in the house of Sir Charles Martin whom we desire to thank for his generous hospitality as well as for his help and advice.

REFERENCES

Birch & György (1936). *Biochem. J.* **30**, 304.
—— —— & Harris (1935). *Biochem. J.* **29**, 2830.
Chick & Copping (1930). *Biochem. J.* **24**, 932.
—— —— & Edgar (1935). *Biochem. J.* **29**, 722.
—— El Sadr & Worden (1940). *Biochem. J.* **34**, 595.
—— Macrae, Martin & Martin (1938). *Biochem. J.* **32**, 844.
—— & Roscoe (1928). *Biochem. J.* **22**, 790.
Copping (1936). *Biochem. J.* **30**, 845.
Cook, Clark & Light (1937). *Proc. Soc. exp. Biol., N.Y.*, **37**, 574.
Edgar & Macrae (1937). *Biochem. J.* **31**, 879.
—— El Sadr & Macrae (1938). *Biochem. J.* **32**, 2200.
El Sadr (1940). *J. Path. Bact.* **50**, 199, *Proc.*
—— Hind, Macrae, Work, Lythgoe & Todd (1939, 2). *Nature, Lond.*, **144**, 73.
—— Macrae & Work (1939, 1). *Biochem. J.* **33**, 611.
Ellinger, Edgar & Lucas (1935). *Chem. Ind. Rev.* **54**, 269.
Goldberger & Lillie (1926). *U.S. Pub. Hlth. Rep.* **41**, 1025.
György (1934). *Nature, Lond.*, **133**, 498.
—— (1938, 1). *Proc. Soc. exp. Biol., N.Y.*, **37**, 732.
—— (1938, 2). *J. Amer. chem. Soc.* **60**, 983.
—— & Eckhardt (1939). *Nature, Lond.*, **144**, 512.
—— Goldblatt, Miller & Fulton (1937). *J. exp. Med.* **66**, 579.
Harris & Folkers (1939). *J. Amer. chem. Soc.* **61**, 1245.
Keresztesy & Stevens (1938). *J. Amer. chem. Soc.* **60**, 1267.
Kuhn & Wendt (1938). *Ber. dtsch. chem. Ges.* **71**, 780.
Lepkovsky (1938). *Science*, **87**, 169.
Lunde & Kringstad (1939). *Hoppe-Seyl. Z.* **257**, 201.
Macrae & Edgar (1937). *Biochem. J.* **31**, 2225.
Morgan, Cook & Davison (1938). *J. Nutrit.* **15**, 27.
Oleson, Bird, Elvehjem & Hart (1939, 1). *J. biol. Chem.* **127**, 23.
—— Elvehjem & Hart (1939, 2). *Proc. Soc. exp. Biol., N.Y.*, **42**, 283.
Tange & Kaneko (1938). *Sci. Pap. Inst. phys. Res., Tokyo*, **35**, 64.

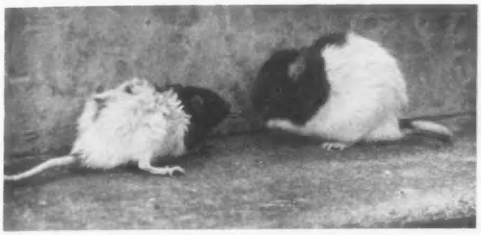


Fig. 4.

Fig. 5.

Fig. 4. (Left) Rat 76♂, body weight 115 g. after 7 months on a synthetic diet containing raw rice starch, with addition of cod liver oil, pure vitamin B_1, riboflavin and purified yeast filtrate factor; (right) litter-mate rat 72♂, body weight 344 g., after the same period on the same diet with addition of pure vitamin B_6 10–15 μg. daily.

Fig. 5. Rat 49, showing dermatitis of ears and nose after 19 weeks' deprivation of vitamin B_6.

(*a*)

(*b*)

(*c*)

(*d*)

Fig. 6. Stages in epileptiform fits developed after 20 weeks on a synthetic diet deprived of vitamin B_6. (*a*) Comatose stage, rat unconscious and flaccid. (*b*) Early stage in recovery of sensation and movement. (*c*) Later stage in recovery; sensation completely regained, while movement restricted to head and forelimbs. (*d*) Recovery nearly complete, rat beginning to move.

LINDA HALL LIBRARY
KANSAS CITY, MO.

71. OCCURRENCE OF FITS OF AN EPILEPTIFORM NATURE IN RATS MAINTAINED FOR LONG PERIODS ON A DIET DEPRIVED OF VITAMIN B_6

By HARRIETTE CHICK, M. M. EL SADR[1] and ALASTAIR N. WORDEN[2]

From the Division of Nutrition, Lister Institute, London, and Roebuck House, Cambridge

(*Received 1 March 1940*)

About 2 years ago Chick *et al.* [1938, 2] reported the occurrence of nervous symptoms in young pigs reared on a synthetic diet and deprived of certain members of the vitamin B_2 complex. The basal diet consisted of purified casein, purified maize starch, cotton seed oil, salt mixture, cod liver oil, with daily supplements of pure vitamin B_1, nicotinic acid and riboflavin. When daily doses of Edgar & Macrae's yeast eluate fraction which contains vitamin B_6 and of their purified yeast filtrate fraction were also given, the pigs developed satisfactorily, though not as well as a control group which received 4 % of yeast in their diet as source of all the B-vitamins. When the filtrate factor was omitted the pigs did badly, became paralysed and died if the missing factor was not given. When the eluate factor was omitted the pigs also showed a subnormal rate of growth and in addition developed a tendency to epileptic fits. The following is the description given.

The fits resembled typical epilepsy as seen in the human being. Without warning the animal ran round the pen in excitement, screamed, then suddenly dropped as if shot. This was followed by a period of tonic spasm with extended legs, passing to a clonic stage with violent jerking of the limbs. A comatose stage succeeded with dry stertorous breathing gradually becoming shallow. A few minutes later the colour of the pig, which had become deathly white, improved, consciousness returned and the animal got up languidly and walked about as before the fit occurred. The duration of the fits was from a few minutes to a quarter of an hour. Between the fits the animals appeared normal. When the yeast eluate fraction was given to the pigs, no more fits occurred.

As far as we are aware, this is the first recorded instance of experimentally produced epilepsy of nutritional origin. Convulsive fits and other nervous symptoms have, however, been described in dogs, pigs, chicks or rats deprived of various members of the B-vitamin group.

Fouts *et al.* [1938] observed the occurrence of hypochromic microcytic anaemia, together with convulsive fits, in puppies about 4 months old reared on a synthetic basal diet of casein, sucrose, crisco, salt mixture, halibut liver oil, vitamin B_1 and riboflavin, to which was added a liver extract which contained the filtrate factor and nicotinic acid. If, in addition, an extract of rice polishings was given, to provide the rat antidermatitis factor, vitamin B_6, there were neither anaemia nor convulsions. Wintrobe *et al.* [1938] described ataxia with queer gait, and clonic, scratch-like, movements of the hind legs, in young pigs receiving a synthetic diet consisting of casein, sucrose, butter, lard, salt mixture, vitamin C and cod liver oil, to which whole yeast was added, at first adequate in amount

[1] Mission Member of the Egyptian University.

[2] Research Student in Animal Health, Ministry of Agriculture.

but gradually reduced to 0·1 g. per kg. body weight. Addition of vitamin B_1 and riboflavin did not prevent the nervous disorder. If the yeast was removed suddenly from the diet, there was prostration, and convulsions occurred [Wintrobe, 1939]. Oleson *et al.* [1939] mention paralysis among the different syndromes observed in rats receiving a synthetic basal diet supplemented with pure vitamin B_1, riboflavin, nicotinic acid, choline and the filtrate obtained after treatment of a liver extract with fuller's earth in acid solution. The rats also suffered from dermatitis; this was cured by pure vitamin B_6, but the paralysis only yielded to an impure preparation of the latter, viz. the eluate obtained from the fuller's earth adsorbate. Finally, Jukes [1939] has reported the occurrence of "spasmodic convulsions", sometimes leading to death, among chicks reared on a diet which included vitamin B_1, nicotinic acid, riboflavin and a preparation of pantothenic acid (filtrate factor); when pure vitamin B_6 was given in addition, there were no fits and growth was satisfactory.

In the course of investigations, extending over many years in this laboratory, of the effects on rats of deprivation of the B_2 group of vitamins and of its separate members, no nervous symptoms have ever been observed until recently. During the series of long-period observations described in the previous paper in this *Journal* [Chick *et al.* 1940], dealing with the skin lesions developed in deficiency of the different B_2-vitamins, fits of an epileptiform nature were observed in rats after long deprivation of the yeast eluate fraction (vitamin B_6).

Experimental

The series of animals studied comprise the 34 rats deprived of the yeast eluate fraction (vitamin B_6) in experiments 1*c*, 2, 3 and 4*a*, of which fuller data are given in Table 2 of the previous paper (p. 587). To this reference should be made for all details, including composition of the basal diets used and the nature and amounts of the supplements given. Fits were seen without exception in all the rats in those groups which received basal diets containing carbohydrate in the form of sucrose or raw starch, and survived upwards of about 4 months. The fits usually occurred some weeks later than the dermatitis; the time of onset varied from 8 to 38 weeks after the animals received the experimental diet and did not normally occur sooner than about 20 weeks (see Table 1), though the rat often became restless and appeared to be hypersensitive some time earlier.

The fits showed the following stages.

i. A violent stage in which the rat would suddenly rush about wildly with protruding eyes, jumping to the floor of the room if not restrained, and leaping up into the air, sometimes uttering cries; this stage usually lasted less than 30 sec. In a few instances the eyes became suffused with blood which drained away through the naso-lachrymal duct. Occasionally the rats urinated during a fit and on one occasion vomiting of stomach contents was observed.

ii. A helpless condition in which there were muscular twitching of the limbs and tonic spasms while the rat lay helpless; sometimes the digits of one of the forepaws became clasped with those of the hind paw on the same side.

iii. A comatose condition when the rat sometimes became unconscious with a slowed and weakened heart beat and absence of corneal reflex.

iv. Gradual recovery, control being regained first of the fore part of the body, head and forepaws, and later of the hind limbs (see Plate 2, fig. 6).

The time taken for the whole fit was usually from 2 to 4 min. and all stages of severity were observed.

In the milder fits stage i might occur alone and the animal come to rest suddenly and remain quiet but conscious; in others stage i might be omitted and

Table 1.* *Incidence of dermatitis and fits in rats maintained for long periods on a synthetic diet supplemented with cod liver oil, pure vitamin B_1, riboflavin and purified yeast filtrate factor and deprived of the yeast eluate factor (vitamin B_6)*

				Incidence of dermatitis		Incidence of fits		
Exp. no.	Litter	Carbohydrate in diet	No. of rats	No. of cases	Time of onset, weeks on diet	No. of cases	Time of onset, weeks on diet	Notes
1*c*, 2	5, 6, 7	Sucrose	6	5	8–11	2	16–20	3 rats killed after 11, 12 and 18 weeks on exp. for histological study,† 1 rat cured with vitamin B_6 at 10 weeks
		Rice starch, raw	5	4	10–24	4	13–38	1 rat died at 16 weeks
		Maize starch, raw	4	2	10–21	4	13–21	
3	8, 9	Sucrose	6	5	8–16	4	17–32	1 rat with severe dermatitis cured with vitamin B_6 at 12 weeks, 1 rat died at 19 weeks
		Rice starch, cooked	5	—	—	—	—	Observed for 30 weeks
		Rice starch, raw	4	2	8	4	8–30	
4*a*	10	Rice starch, raw	4	3 1	8–9 30	3	21–28	1 rat died at 14 weeks
4*b*‡	10	Rice starch, raw	4	—	—	—	—	All healthy after 35 weeks

* Reference should be made to Table 2 of the preceeding paper which deals with the same series of animals.
† By Dr El Sadr. ‡ Addition of pure vitamin B_6, 10–15 μg. daily.

the animal, without warning, suddenly become helpless with twitching paws. In others, again, recovery began immediately after stages i and ii, and there was no comatose stage.

When a fit was over, the rat usually remained very quiet for a long time, for an hour or more, with protruding eyeballs, and the hypersensitive condition persisted. A second fit could sometimes be induced if, for example, the tail were slightly pinched. Only on one occasion did an animal die in a fit.

The rats also showed minor nervous signs when fits did not occur. Such were occasional spasm of the ear muscles and eyelids and twitching of the shoulders when the daily doses were dropped into the mouth. Sometimes a rat would struggle and lunge to get free from one's hand when being dosed, which is quite unusual with rats which are accustomed to being handled every day and are quite tame.

Sometimes the fits were of daily occurrence and the slightest external stimulus seemed to precipitate them. Three such rats were sacrificed for bacteriological examination, in order to find out if the occurrence of the fits could be attributed to infection by a pleuro-pneumonia like organism, as was found to be the case with the "rolling disease" of mice [Findlay *et al.* 1938]. The examinations were carried out by Dr Emmy Klieneberger, to whom our thanks are due, but no evidence of a causal infective agent was found. Histological examination of the central nervous system by Dr J. G. Greenfield gave negative results (see Addendum).

In many instances, in a rat which had suffered from fits and had remained on the same diet, the fits ceased to occur but the animal remained nervous and

excited and showed protruding eyeballs. In such cases a fit could be induced sometimes by handling or stroking for 15–30 min. Sometimes attempts to obtain a drop of blood from the tail for haematological examination caused a fit to take place, and fits were also precipitated when rats were placed in an unaccustomed situation in order to take a photograph. It was very common, before a fit took place, to notice chattering of the teeth and also to observe the rat rubbing its nose and cheeks with its forepaws, as if these places were irritable.

The fits were somewhat similar in character to those observed by McCollum and his collaborators in rats deprived of magnesium [see Duckworth, 1939]. The description of the convulsions caused by Mg deficiency in the rat given by McCollum *et al.* [1939] shows a striking similarity to the fits described here. However, our basal diet contained 0·07 % Mg and consequently our animals received an adequate supply.

Relation of fits to deprivation of vitamin B_6

The rats which developed fits were all deprived of the yeast eluate factor, that is of vitamin B_6 [see El Sadr *et al.* 1939, 2]. We have so far no evidence of the presence of any other dietary essential for the rat in the preparation of this factor which was used [Edgar *et al.* 1938]. It may have contained nicotinamide or nicotinic acid [Macrae & Edgar, 1937, p. 2229, n.], but there is no evidence that the rat requires either of these compounds [Macrae & Edgar, 1937; Chick *et al.* 1938, 1]. Of the 15 rats in Exp. 3, 7, distributed over the 3 groups receiving the 3 different types of carbohydrate, received 1 mg. nicotinic acid daily; these behaved in no way differently from the 8 which had no nicotinic acid.

The supposition that the fits were caused by lack of vitamin B_6 is also confirmed by the following facts. In Exp. 4 (*b*) the 4 rats which received 10 to 15 μg. pure vitamin B_6 daily showed no signs of fits, in contrast to their litter-mates deprived of it. They also showed no skin affection and became healthy well-grown rats. After 33 weeks the males and females weighed respectively about 330 and 240 g., whereas their litter-mates deprived of vitamin B_6 weighed only about 110 g. irrespective of sex (see Fig. 3 of the previous paper, this *Journal*, p. 590).

The curative effect of pure vitamin B_6, 15 μg. daily, was tried on 3 rats (Nos. 35, 36 and 51) suffering from fits after deprivation of eluate factor for periods of 15, 20 and 22 weeks, respectively. Of these one had no more fits; two continued to have them only during the first 2 weeks after the cure began; all three were observed for 2–3 months after the vitamin B_6 was given. Two of the above rats (Nos. 35 and 51) were suffering also from dermatitis when the cure with vitamin B_6 was given; these and others suffering from very severe dermatitis showed swift cure of the skin condition after administration of 15 μg. vitamin B_6 daily.

Influence of carbohydrate in the diet

The results in Table 1 show that whereas fits occurred in all the rats receiving raw maize or rice starch, no fits occurred in the group in Exp. 3 which received the cooked rice starch, nor was there any dermatitis. The protection afforded by starch, as compared with sucrose, against the dermatitis caused by deficiency of vitamin B_6 is discussed in the previous paper (p. 591) and the suggestion is there made that it is due to traces of vitamin B_6 which adhere to the starch, and in some way become more available to the rat after cooking.

If this explanation be the true one it offers another argument for relating the occurrence of the fits to deprivation of vitamin B_6. It must be admitted, however,

that the diet given was deficient in other respects than in vitamin B_6. It is known that, in addition to riboflavin, yeast filtrate factor and vitamin B_6, there are present in whole yeast and in an extract of whole liver at least two other, at present unknown, water-soluble substances, which appear to be essential for the optimum nutrition of the rat (El Sadr *et al.* 1939, 1]. These unknown materials were probably absent from the diets given in this work, although traces may have been present as impurities in the starch and casein, and it must be admitted that the growth rates when vitamin B_6 was added to the diet fell somewhat short of the optimum (see Exp. 4*b*, Fig. 3 of the previous paper). Further, although successful pregnancies followed matings in this group, the litters were not reared. In any case, however, there is no evidence that the fits were caused by deprivation of either of the above unknown factors and there is much positive evidence that they were due to deprivation of vitamin B_6.

It will seem very strange that the epileptic fits described in this paper should not have been observed previously in any of the large numbers of rats deprived of vitamin B_6 studied in this laboratory. In the present case they occurred in every one of the 21 rats observed for 4 months or longer on the purified synthetic diet containing sucrose or raw purified starch as source of carbohydrate. While it is true that in the past a cooked basal diet containing starch as carbohydrate has usually been employed, recently sucrose has been used more frequently. The experiments of Series I in the previous paper are an example and here no fits were observed in rats deprived of yeast eluate fraction. None of these animals, however, was observed for a longer time than 14 weeks and in most cases the period was shorter.

The results of these experiments with rats fall into line with the previous ones on pigs by Chick *et al.* [1938, 2], alluded to above. The basal diets and the supplements given were similar. At the time when the experiments on pigs were carried out pure vitamin B_6 was not available, but preparations of the yeast eluate factor were found to be curative and it is reasonable to conclude that deprivation of vitamin B_6 was the immediate cause of the fits in the pigs as in the rats.

A connexion between deprivation of vitamin B_6 and the occurrence of the convulsive fits and other nervous symptoms in puppies, pigs, rats and chicks, described above on p. 595, is not so clear. In some of the observations, as for example those of Wintrobe and his colleagues on the pig, the diet was deficient in other known members of the vitamin B_2 complex; in others, as in those of Jukes on the chick, the basal diet was not composed of purified foodstuffs, although in this case pure vitamin B_6 was found to be preventive of the convulsions which occurred.

Summary

1. Fits of an epileptiform nature were observed in rats maintained for long periods, 4 to 5 months and over, on a purified synthetic diet supplemented with cod liver oil, pure vitamin B_1, riboflavin and purified yeast filtrate factor.

2. The fits were prevented and cured by administration of pure vitamin B_6, 10–15 μg. daily, and it is concluded that they were caused by deprivation of this vitamin.

3. No fits occurred when purified rice starch was the carbohydrate in the diet and the diet was cooked with water; it is suggested that this starch retains traces of vitamin B_6 which become more readily available to the animal after the starch has been cooked.

4. The nature of these fits in rats and the circumstances in which they were developed show the closest resemblance to the epileptic fits previously observed

in young pigs. The basal diets and the supplements given were similar in the two cases. It is reasonable to conclude that in the pigs they were also caused by deficiency of vitamin B_6.

Our thanks are due to Mrs C. E. Work and to Mr G. Flynn for assistance in the care of the experimental animals, to the former especially for her help during September when the rats made a temporary stay at the Lister Institute, Elstree, before they were finally settled at Roebuck House, Chesterton, Cambridge, through the hospitality of Sir Charles Martin, to whom we wish to express our gratitude.

We are also indebted to Dr S. Lepkovsky for a gift of pure vitamin B_6 and to Roche Products Ltd. for riboflavin and pure vitamin B_1 (aneurin).

REFERENCES

Chick, Macrae, Martin & Martin (1938, 1). *Biochem. J.* **32**, 844.
—— —— —— —— (1938, 2). *Biochem. J.* **32**, 2207.
—— —— & Worden (1940). *Biochem. J.* **34**, 580.
Duckworth (1939). *Nutrition Abstracts and Reviews*, **8**, 841.
Edgar, El Sadr & Macrae (1938). *Biochem. J.* **32**, 2225.
El Sadr, Hind, Macrae, Work, Lythgoe & Todd (1939, 1). *Nature, Lond.*, **144**, 73.
—— Macrae & Work (1939, 2). *Biochem. J.* **33**, 611.
Findlay, Klieneberger, MacCallum & Mackenzie (1938). *Lancet*, **135**, 1511.
Fouts, Helmer, Lepkovsky & Jukes (1938). *J. Nutrit.* **16**, 197.
Jukes (1939). *Proc. Soc. exp. Biol., N.Y.*, **42**, 180.
McCollum, Orent-Keiles & Day (1939). Newer Knowledge of Nutrition, 5th ed. New York.
Macrae & Edgar (1937). *Biochem. J.* **31**, 2225.
Oleson, Bird, Elvehjem & Hart (1939). *J. biol. Chem.* **127**, 23.
Wintrobe (1939). *Amer. J. Physiol.* **126**, 375.
—— Mitchell & Kolb (1938). *J. exp. Med.* **68**, 207.

ADDENDUM

By Dr J. G. Greenfield

Results of histological examination of the tissues of rats suffering from fits

These rats, Nos. 40, 50 and 54, had been maintained on the diet deprived of vitamin B_6 for 31, 21 and 30 weeks, and had suffered from fits for 11, 5 and 13 weeks, respectively.

The brain and spinal cord and sciatic nerves were examined by the Marchi-Busch and Nissl methods.

The nerve cells throughout the cerebral nervous system were healthy, and there was no evidence of any falling out of nerve cells in the *Cornu Ammonis* or elsewhere. No Marchi degeneration was found in the medulla, spinal cord or sciatic nerves.

In the lungs of rat 40 there was considerable thickening of the alveolar septa in many places with fibro-cellular tissue. In some areas a few foamy cells were present in the alveoli. The bronchial walls were healthy. In the lungs of rat 54 the condition was similar but in places seemed to be more severe and acute, in that the alveoli were almost completely obliterated and more polymorphonuclear leucocytes were present in the alveolar septa. Such changes as the above are commonly present in rats maintained for long periods on artificial diets and would appear to bear no relation to the specific deficiency in this case.

72. THE ESTIMATION OF RIBOFLAVIN

PART 1. A NEW BIOLOGICAL METHOD

By M. M. EL SADR, T. F. MACRAE and C. ELIZABETH WORK

From the Division of Nutrition, Lister Institute, London

PART 2. THE ESTIMATION OF RIBOFLAVIN IN MILK: COMPARISON OF FLUORIMETRIC AND BIOLOGICAL TESTS

By K. M. HENRY, J. HOUSTON and S. K. KON

From the National Institute for Research in Dairying, University of Reading

PART 3. STATISTICAL ANALYSIS OF THE DATA

By J. O. IRWIN

Of the Medical Research Council's Statistical Staff

(*Received 1 March 1940*)

PART 1

The importance of riboflavin in the nutrition of the rat has been recognized for some years. Rats given a diet deficient in this factor cease to grow [Edgar *et al.* 1937] and later develop severe pathological symptoms [Day *et al.* 1938; Bessey & Wolbach, 1939; El Sadr, 1940].

It is comparatively recently that riboflavin has been proved to be an essential nutrient of other mammals. Sebrell & Onstott [1938] and Street & Cowgill [1939] showed that dogs, when given a diet deficient in riboflavin, developed symptoms which rapidly led to collapse and death; these symptoms were prevented by addition of riboflavin to the diet and certain sick animals were cured by administration of this compound. Margolis *et al.* [1939] demonstrated that a black-tongue-producing diet supplemented by nicotinic acid would not maintain dogs in good health unless further supplements of aneurin and riboflavin were given. Hughes [1939] has indicated that riboflavin is required for the well-being of the pig.

Sebrell & Butler [1938] fed a restricted diet, used previously by Goldberger & Tanner [1925], to a group of women and observed the development of lesions at the corners of the mouth; these symptoms were rapidly cured by administration of riboflavin but not of nicotinic acid. Oden *et al.* [1939] found a similar condition occurring naturally in Georgia; again remission of the symptoms was effected by administration of riboflavin. It is possible that some pellagra-producing diets are deficient in riboflavin as well as in nicotinic acid and aneurin, since nicotinic acid alone or with aneurin has not always been found to cure pellagra [Schmidt & Sydenstricker, 1938; Ghalioungui & Hanna, 1938]. According to

Vilter *et al.* [1939] patients who had been cured of pellagra by administration of nicotinic acid and aneurin, and were maintained on the pellagra-inducing diet and the above supplements, later developed certain nervous and digestive symptoms which were cured by administration of riboflavin.

Since the dietary value of riboflavin is established, the estimation of this vitamin in foodstuffs is a matter of importance; many methods have already been described, some biological and others physico-chemical. The latter methods (see Karrer [1939]) at present cannot be considered always to be reliable, but have the advantage of rapidity. The usual procedure is to extract the riboflavin either with aqueous alcohol or acetone, purify the extract by various methods and finally to estimate, fluorimetrically or colorimetrically, the riboflavin in the extract. In some cases the riboflavin is converted into lumiflavin which is estimated colorimetrically. Errors may arise from incomplete extraction of riboflavin from the foodstuffs, losses during the purification of the extract, interference by coloured or fluorescent impurities and, when conversion into lumiflavin is employed, losses due to incompleteness of such conversion. In certain cases the physico-chemical methods may yield satisfactory results but many careful experiments of comparison with satisfactory biological methods must be carried out before their adoption becomes justifiable.

In the biological methods for estimation of riboflavin which have been described, the growth of rats is usually taken as a criterion of biological activity. The provision of a basal diet free from riboflavin but containing all other essential nutrients has hitherto not been attained, and most of the diets which have been used are deficient in at least one factor of the vitamin B_2 complex besides riboflavin. Any growth response obtained by the addition of a foodstuff to these diets may be due to the supplementary effect of riboflavin or of the other nutrients in which the particular basal diet is deficient, or, more probably, to the combined effects of riboflavin and the other factors. Such methods of estimation must tend to give too high values for the riboflavin content of foodstuffs.

Many of the rat growth methods of estimation of riboflavin are based on the method of Bourquin & Sherman [1931]. The diet employed by these authors contained, as source of B-vitamins other than riboflavin, an 80 % alcoholic extract of wheat evaporated on to corn starch. Booher [1932], Bessey & Wolbach [1939] and Sherman & Langord [1938] used this diet. György *et al.* [1934] observed increased growth rates when the diet was supplemented either with a fuller's earth filtrate of yeast extract or a Peters's preparation of vitamin B_4 [Kinnersley *et al.* 1933]. György [1935] reported a considerable variation in the content of B_2-vitamins of different samples of wheat. Booher *et al.* [1934] and Copping [1936] showed that, for a given dose of riboflavin, the growth rate could be increased by raising the level of the wheat extract in the diet. Carlsson & Sherman [1938] pointed out the possibility of a deficiency of some members of the vitamin B_2 complex occurring in the basal diet, and suggested that food products such as liver, milk and muscle may contain unknown growth-promoting factors which are absent from the basal diet. It appears therefore that the Bourquin-Sherman diet is not entirely satisfactory for the estimation of riboflavin, as it does not contain adequate amounts of all the members of the vitamin B_2 complex other than riboflavin. This is not surprising as the diet was originally intended to provide vitamin B_1 free from what was then known as vitamin B_2 or vitamin G. The Bourquin and Sherman "unit" of riboflavin potency was defined as that amount which, when given daily, maintained an average growth rate in rats of 3 g. weekly. This "unit" was later calculated to be equivalent to 2–2·5 μg. of riboflavin [Bessey, 1938]. The use of a standard growth rate as a reference unit is to

be criticized on theoretical grounds and in any case a weight increase of 3 g. weekly is too low, since at this level the margin of error is large.

Day *et al.* [1937] employed a diet in which an 80% alcoholic extract of rice polishings provided the riboflavin-free supplement, and claimed that their method measured riboflavin when the growth rate was less than 10 g. weekly. Ansbacher *et al.* [1936] and Cook *et al.* [1937] used as sources of additional B-vitamins concentrates prepared from rice polishings, and Supplee *et al.* [1939] used these concentrates after they had been autoclaved. Lindholm [1938], in the construction of a standard response curve to be used for the estimation of riboflavin in foodstuffs, provided the other members of the vitamin B_2 complex as an aqueous extract of liver treated with fuller's earth at *p*H 4; fuller's earth is known to remove vitamin B_6 (adermin) as well as riboflavin from solution. Lunde *et al.* [1939] fed the standard "Peters's eluate" preparation of vitamin B_1 which is known to contain some vitamin B_6, but which cannot supply adequate amounts of all B_2-vitamins. Euler *et al.* [1934] used as source of additional B_2-vitamins an extract of yeast which had been treated with frankonite.

It is improbable that any one of the above diets contained adequate amounts of all B_2-vitamins other than riboflavin and many probably contained appreciable amounts of riboflavin due to the incorporation of inadequately purified casein. Supplee *et al.* [1936] effected progress in the purification of the basal diet by showing that when casein is washed with salt solution at its isoelectric point, riboflavin is demonstrable in the washings even when the casein had been washed previously with alcohol and acetic acid.

Other biological methods for the estimation of riboflavin have been described. Jukes [1937] used, as criterion of riboflavin activity, the growth rate of chicks fed on a grain diet supplemented by a concentrate prepared from rice polishings. Snell & Strong [1939] employed the growth-promoting action of riboflavin for *Lactobacillus casei*. The latter method is quick and by its use concentrations of riboflavin as low as 0·05 μg. per 10 ml. of medium can be estimated, but it involves the preliminary extraction of riboflavin from the foodstuffs; this is known to be difficult to carry out quantitatively in certain cases.

In this laboratory it is considered that riboflavin may best be estimated by using the growth of young rats as the criterion of biological activity. In earlier experiments [Edgar *et al.* 1937], a growth response curve to graded doses of riboflavin was constructed with the use of a diet which contained yeast fuller's earth filtrate as source of B_2-vitamins other than riboflavin. It was soon realized that this diet was deficient in vitamin B_6 and probably in other factors, as well as in riboflavin, and was therefore not suitable for the estimation of riboflavin in foodstuffs.

In further experiments, designed to yield a method for the biological estimation of riboflavin, various supplements from yeast and liver were investigated as sources of B_2-factors other than riboflavin. Treatment of aqueous extracts of whole liver with norite charcoal was found to remove the riboflavin quantitatively, and the resulting filtrate contained adequate amounts of all other B_2-vitamins recognized to be essential for the rat. With this supplement, a growth response curve to graded doses of riboflavin has been constructed. The application of this method to the estimation of riboflavin in foodstuffs is now being carried out in this laboratory and the results obtained will be published later. We submitted details of our method with the liver charcoal filtrate to Dr S. K. Kon and his collaborators, who have not only repeated our experiments and constructed a growth response curve similar to our own, but have also applied the method to the estimation of riboflavin in certain milk products and have compared the

resulting values with those obtained by fluorimetric means. Part 2 of this paper gives the results obtained. Dr J. O. Irwin has analysed the results obtained in both laboratories; his report is contained in Part 3.

Methods and results

The animal technique employed was essentially that described previously [Edgar *et al.* 1938]. Young rats, whose mothers had received the stock breeding diet except during the last week of lactation when no yeast was fed, were weaned at 21 days; they weighed 40–50 g. They received the vitamin-deficient ration employed in this laboratory, consisting of casein 100,[1] rice starch 300, cottonseed oil 60, lard 15, salt mixture (McCollum's No. 185) 25 and water 500; the diet was cooked by steaming for 3 hr. Each rat received daily supplements of 0·08–0·1 ml. cod liver oil and 10–15 μg. aneurin chloride hydrochloride. Male rats only were used.

The animals, after gaining slightly in weight for about 7–10 days, reached a constant weight and were then given the test dose of riboflavin together with the supplements described below. The animals from various litters were divided as equally as possible amongst the groups receiving the different doses of riboflavin. The growth rate was observed for a period of 4 weeks.

A. *Growth response of rats to graded doses of riboflavin when additional B_2-vitamins are supplied as a crude yeast fuller's earth filtrate fraction and yeast fuller's earth eluate fraction (Table 1, Group A; Fig. 1)*

When the first curve of growth response to graded doses of riboflavin was made in this laboratory [Edgar *et al.* 1937] the rats received yeast fuller's earth filtrate as source of B_2-vitamins other than riboflavin. It was then realized [Edgar & Macrae, 1937] that these animals were deficient in what was then termed "yeast eluate factor", now known to be identical with vitamin B_6 [El Sadr *et al.* 1939, 2]. A further response curve therefore was made from results of tests in which each animal received the B_2-vitamins other than riboflavin as daily doses of (1) crude yeast fuller's earth filtrate = 1 g. dry yeast [Edgar *et al.* 1937] and (2) yeast fuller's earth eluate = 2 g. dry yeast [Edgar & Macrae, 1937]. The presence in the diet of the yeast eluate fraction caused a considerable increase in the growth rates at the various levels of riboflavin dosage (Table 1, Group A; Fig. 1), the maximum growth response being 30·4 g. weekly compared with 18 g. weekly observed when no eluate fraction was given [Edgar *et al.* 1937]. In these experiments the casein was not purified by washing with salt solution.

B. *Growth response of rats to graded doses of riboflavin when additional B_2-vitamins are supplied as a purified yeast fuller's earth filtrate fraction purified by extraction with amyl alcohol and yeast fuller's earth eluate fraction (Table 1, Group B; Fig. 1)*

The yeast fuller's earth filtrate was purified by the method described by Edgar *et al.* [1938]; each rat received amounts equivalent to 2 g. dry yeast daily of both the filtrate and eluate fractions. The casein in the basal diet was purified

[1] Casein used was "Glaxo ashless extracted". In later experiments this was washed by the following method: 2·5 kg. were stirred for 30 min. with 30 l. tap water containing 600 g. NaCl and 30 ml. glacial acetic acid. After an hour the supernatant liquor was poured off. The washing was repeated 6 times. The casein was then pressed as dry as possible on Büchner funnels, suspended in 96% alcohol, filtered and finally dried on open trays in a current of air.

Table 1

Growth response of young male rats to increasing doses of riboflavin. Group A animals received ashless extracted casein diet with crude yeast filtrate fraction (=1 g. dry yeast daily) and yeast eluate fraction (=2 g. dry yeast daily) as sources of B_2-vitamins. Group B animals received washed casein diet with yeast filtrate fraction purified by amyl alcohol extraction (=2 g. dry yeast daily) and yeast eluate fraction (=2 g. dry yeast daily) as source of B_2-vitamins.

	Group A		Group B	
Daily dose of riboflavin μg.	No. of rats	Av. weekly wt. increase during 4 wk. period (g.)	No. of rats	Av. weekly wt. increase during 4 wk. period (g.)
0	4	7·5	2	3·5
3	5	10·6	2	7·2
6	5	13·9	2	10·0
12	7	19·3	2	14·1
25	5	20·6	—	—
37	2	27·6	—	—
50	9	30·4	6	20·4

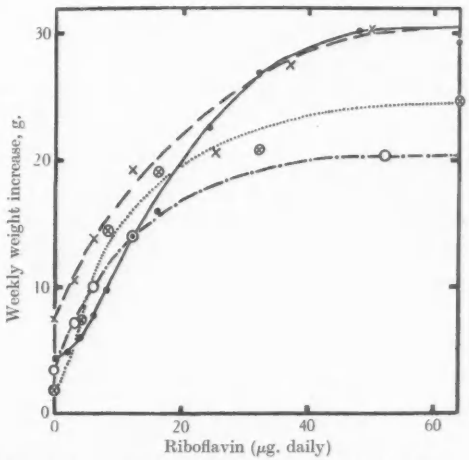


Fig. 1. Response of rats to increasing doses of riboflavin. Data of El Sadr *et al.* — — (Exp. points ×) rats received crude yeast filtrate fraction and yeast eluate fraction. ·—·— (Exp. points ○) rats received purified yeast filtrate fraction and yeast eluate fraction. ——— (Exp. points •) rats received liver charcoal filtrate. Data of Kon & Henry. ……(Exp. points ⊗) rats received liver charcoal filtrate. The curves have been fitted by eye to display the observed points more clearly.

by washing with salt solution. The growth rates observed at the various dose levels of riboflavin (Table 1, Group B; Fig. 1) were considerably less than the corresponding values obtained using the less pure fractions. The growth rate of 3·5 g. weekly of rats receiving no added riboflavin may be compared with the previous value of 7·5 g. weekly (Table 1, Group A) and suggests that the further purification of the diet had resulted in the removal of appreciable amounts of riboflavin. The maximum growth rate of 20·4 g. weekly, observed when optimal amounts of riboflavin were administered, indicated, however, that the diet was deficient in at least one member of the vitamin B_2 complex additional to riboflavin; this must have been lost in the purification processes. This possibility has been substantiated by further work [El Sadr *et al.* 1939, 1].

C. *Growth response of rats to graded doses of riboflavin when additional B_2-vitamins are supplied as a liver extract treated with norite charcoal (Table 2; Fig. 1)*

The diet finally adopted for the biological estimation of riboflavin contained casein which had been purified by washing with salt solution, while additional B_2-vitamins were supplied by a liver charcoal filtrate.

Preparation of liver charcoal filtrate. 1 l. of liver extract, prepared by extraction of 6 kg. of whole liver with 50 % aqueous acetone and subsequent removal of the acetone *in vacuo*, was adjusted to *p*H 5 with HCl and 36 g. norite charcoal (Messrs Hopkin & Williams) were added. After stirring for 30 min. the charcoal was allowed to sediment for 2 hr. and was then removed by filtration. The treatment was repeated with an equal amount of norite charcoal, and the final filtrate was fed to the rats without further treatment. Each rat received 1 ml. (=6 g. fresh liver) daily.

With this basal diet the growth rates varied consistently with the dose of riboflavin as shown in Table 2 and Fig. 1. The maximum growth rate of 30·2 g. weekly was similar to that observed when crude yeast fuller's earth filtrate and

Table 2. *Data of El Sadr* et al.

Growth response to increasing doses of riboflavin of male rats receiving purified casein in the diet and whole liver extract treated with norite charcoal =6 g. fresh liver daily.

Daily dose μg.	No. of animals	Mean observed increase in wt. per week (over 4 weeks) g.	Calculated values		
			Equation (1)	Equation (2)	Equation (3)
0	8	4·34	—	—	—
2	6	4·92	0·52	5·50	—
4	6	6·04	6·49	4·83	—
6	6	7·88	9·99	7·31	6·59
8	5	9·80	12·47	9·94	9·88
12	8	14·12	15·96	14·44	14·52
16	8	16·03	18·44	17·89	17·81
24	7	22·75	21·94	22·65	22·46
32	8	26·75	24·42	25·68	25·75
48	10	30·22	27·91	28·98	—
64	10	29·28	30·39	30·35	—

yeast fuller's earth eluate fractions were given as sources of additional B_2-vitamins (Table 1, Group A); however, the lower growth rate of 4·3 g. weekly given by the basal diet containing the liver supplement without added riboflavin suggested that this diet was less contaminated with riboflavin.

The liver charcoal filtrate described above contains adequate amounts of all the recognized members of the vitamin B_2 complex required by the rat except riboflavin. It is known that the factor we have named liver filtrate factor is adsorbed to some extent by norite charcoal [Macrae *et al.* 1939] but adequate amounts appear to be present in the filtrate as no increased growth rate was observed after addition of purified concentrates of this factor. It has long been recognized that norite charcoal is not a good absorbent for vitamin B_6. The high growth rates observed when optimal amounts of riboflavin were administered prove that the less well characterized members of the vitamin B_2 complex were present in the charcoal filtrates. It is true that somewhat better growth rates were obtained with untreated extract of whole liver as source of all B_2-vitamins than with the liver charcoal filtrate and optimal amounts of riboflavin. The difference, however, is so slight that it is considered improbable that

the presence of B-vitamins other than riboflavin in foodstuffs tested would appreciably affect the growth rate of rats receiving the above liver charcoal filtrate in their basal diet. We therefore consider that this basal diet and procedure may be satisfactorily used for the estimation of riboflavin in all foodstuffs. The basal diet has the advantage of being easily prepared.

Statistical examination shows that the usual method of biological estimation, involving the administration of both unknown and standard at two dose levels may be employed.

Norite charcoal has been found to remove riboflavin from the other B_2-vitamins of yeast extracts. In the above method of riboflavin estimation, therefore, yeast charcoal filtrate may be substituted for liver charcoal filtrate.

SUMMARY

Treatment of extracts of liver or yeast with norite charcoal yielded a filtrate which, while deficient in riboflavin, contained all other known constituents of the vitamin B_2 complex. Young rats when given a diet complete in other respects and containing liver charcoal filtrate as source of B_2-vitamins showed graded growth response to graded doses of riboflavin. A method is therefore suggested whereby the riboflavin contained in foodstuffs may be determined by employing one of these filtrates as the riboflavin-free supplement to the diet.

We express our thanks to Dr H. Chick for her advice and criticism. The whole liver extract used in the above experiments was supplied by Messrs Glaxo Laboratories Ltd., and the aneurin and riboflavin by Messrs Roche Products Ltd. We are most grateful to these firms for their continued help.

PART 2

A fluorimetric assay of the riboflavin content of milk has been in use for some time in this laboratory [Henry *et al.* 1939], but without parallel biological tests it had not so far been possible to judge how reliable it was.

Dr Macrae very kindly kept us informed of the developments of the biological method described in Part 1 of this paper and we were able to carry out two series of tests on milk in time for a joint publication.

EXPERIMENTAL

1st experiment

A. *Biological tests on rats.* 1. Preparation of animals. Mothers with young were changed on the 16th day of lactation from the stock colony diet [Folley *et al.* 1938] to a similar diet from which yeast, liver and milk had been removed. Males only were used for these tests. They were weaned at 21 days, weighing 30–50 g., placed in individual cages and given the riboflavin-deficient diet as described in Part 1. They received daily a solution containing 10μg. of aneurin chloride hydrochloride and 2 drops of cod liver oil. There was hardly any growth on this diet and after 7–10 days, when the animals were either stationary in weight or losing slightly, they were divided into groups of 8, the allowance of aneurin was increased to 15μg. daily and each rat was given in addition 0·5 ml. (=6 g. of fresh liver) of the liver charcoal filtrate prepared as described in Part 1, and concentrated in a current of air at 40°.

One control group received no further additions, five other groups received respectively 4, 8, 16, 32 and 64 μg. daily of pure synthetic riboflavin ("Roche") which we found by fluorimetric tests to be equal to the highest commercially available grade of the natural substance. Two more groups received respectively 0·9 and 1·8 g. daily of spray-dried full cream milk to supply riboflavin at presumptive levels of approximately 10 and 20 μg.

2. Riboflavin standard. A stock solution containing 100 μg./ml. was made up in 50 % methyl alcohol. From this the necessary quantity was removed daily, evaporated *in vacuo* almost to dryness and made up in water at *p*H 2 so that the solution contained 40 μg./ml. The strength of this solution was checked against a fluorimetric standard each day before feeding. The solutions were kept in dark bottles.

3. Milk. The dried milk was dissolved each day in water so that 0·9 g. was contained in 5 ml. Fluorimetric tests were done at weekly intervals. Double doses of all supplements were fed on Saturdays to avoid Sunday dosing. The test lasted 4 weeks.

B. *Fluorimetric tests.* The milks were prepared for assay by the method of Emmerie [1938] and measurements were carried out in the outfit described by Henry *et al.* [1939].

C. *Results.* The mean gains in weight of the experimental animals are shown in Table 3 and Fig. 1. The riboflavin content of milk calculated from these data and the results of fluorimetric tests are given in Table 5; the value of 15·2 μg. per g. was obtained by calculating a pooled slope for test and standard [Irwin, 1937]. When the smaller and larger doses were interpreted separately with reference to the standard curve, results of 12·2 μg. per g. and 19·1 μg. per g. were obtained. These do not differ significantly, but there is a discrepancy between the higher value and the result of the fluorimetric test.

Table 3. *Data of Henry* et al. (*1st experiment*)

Daily dose of standard (μg.)	0	4	8	16	32	64
No. of animals	8	8	8	8	8	8
Observed mean increase in wt. per week (over 4 weeks) (g.)	1·84	7·31	14·59	19·09	20·94	24·94
Daily dose of spray-dried milk (g.)	0·9	1·8				
No. of animals	8	8				
Observed mean increase in wt. per week (over 4 weeks) (g.)	15·12	21·97				

2nd experiment

A. *Biological tests on rats.* 1. Preparation of animals. The preliminary procedure was the same as in the first experiment. The rats weighed 34–48 g. at weaning. Again, males only were used in groups of eight. Riboflavin was fed at levels of 4, 8, 16 and 32 μg. daily.

2. Riboflavin standard. An aqueous solution at *p*H 2 containing 40 μg. in 1 ml. was freshly made every 3 days. It was checked daily by fluorimetric tests.

3. Milk. The same sample of full cream spray-dried milk was used, 0·5 and 1·0 g. were fed corresponding to presumptive levels of intake of 5 and 10 μg. In addition an evaporated milk was also tested. Of this 2 and 4 g. (0·58 and 1·16 g. on the dry basis) were given daily diluted with water to 5 and 10 ml. respectively. The presumptive levels of feeding were in this case approximately 6 and 12 μg. of riboflavin.

B. *Results.* The results are given in Tables 4 and 5. With one exception the control animals gained little weight. One animal however gained 6 g. in the first fortnight of the experiment and 46 g. in the second. There was no obvious

Table 4. *Data of Henry* et al. (*2nd experiment*)

Daily dose of standard (μg.)	0	4	8	16	32
No. of animals	7	7	8	8	8
Observed mean increase in wt. per week (over 4 weeks) (g.)	0·48	9·14	14·91	19·06	22·31

	(*a*)		(*b*)	
Daily dose of:				
(*a*) spray-dried milk	0·5	1·0		
(*b*) evaporated milk (g.)			2·0	4·0
No. of animals	8	8	8	8
Observed mean increase in wt. per week (over 4 weeks) (g.)	9·78	15·88	10·84	16·28

Table 5. *Results calculated from the data of Henry* et al.

	Biological test			
		Limits of error (%)		Fluorimetric test result
Experimental material	Result μg./g.	($P = 0·95$)	($P = 0·99$)	μg./g.
(*a*) Spray-dried milk	15·2	75–133	69–145	10·0
(*b*) Spray-dried milk	9·3	76–131	70–142	10·3
(*c*) Evaporated milk	2·6	78–128	72–139	2·9

explanation of this behaviour but its abnormal increase in weight has been omitted from the mean. One animal, receiving 4 μg. of riboflavin, died of purulent pneumonia on the 23rd day of the experiment.

The results show that when milk is fed at lower levels, yielding weight gains of 10–15 g. a week, biological findings are in good agreement with fluorimetric tests.

Discussion

It is of interest to compare results obtained in the present study with other reports of biological and especially of simultaneous biological and chemical assays of riboflavin in milk. Kramer *et al.* [1938] applied the Bourquin-Sherman [1931] method to milk and obtained a value of 2 μg. per g. for winter herd milk. Whitnah *et al.* [1938] assayed milk from the same farm fluorimetrically and obtained, before cows went out to pasture, values varying according to breed from 1·17 to 1·73 μg. per g. In a later paper Kramer *et al.* [1939] reported an average difference of only 10 % between the results of biological (Bourquin-Sherman method) and fluorimetric riboflavin estimation on milk. Snell & Strong [1939] obtained by biological tests, presumably by the Bourquin-Sherman [1931] method, a value of 17 μg. per g. for skim milk powder and an identical value by their microbiological test with *Lactobacillus casei*. Hodson & Norris [1939] report values of 17–22 μg. per g. for skim milk powder by fluorimetry and very similar figures using the microbiological test of Snell & Strong [1939]. Finally, Lunde *et al.* [1939] quoted a biological value for fresh milk of 2·4 μg. per g. and 2·7 μg. by fluorimetry. The agreement in all these tests is excellent but it should be noted that the biological tests consisted mostly in feeding only one level of the standard and one of the unknown.

Summary

1. The biological method of assay of riboflavin described by El Sadr *et al.* (Part 1) has been compared with fluorimetric tests for full cream spray-dried milk and for evaporated milk.

2. Good agreement was found when the milks were fed at levels supplying up to 10 μg. daily. For a higher level the agreement was not so satisfactory.

PART 3

(1) *Data of El Sadr et al. (Tables 2 and 6; Fig. 2)*

Table 2 shows the observed mean increase in weight per week over a period of 4 weeks for the negative controls and each of the 10 doses. The best fitting straight line relating response to the logarithm of the dose is

$$y = -5{\cdot}458 + 19{\cdot}849x \qquad \text{.....(1)}$$

where y = response, and x = log dose.

Table 6. *Slopes and their standard errors; variance ratios for the linearity test*

	Slope	Standard error	Variance ratio	5% point of variance ratio
El Sadr *et al.*:				
Standard, all points	19·85	0·76	10·4	2·1
Central, 6 points	26·36	1·40	2·5	2·6
Henry *et al.*:				
Exp. 1 (*a*) Standard	13·82	1·10	2·7	2·9
(*b*) Spray-dried milk	22·73	3·87	—	—
(*a*) and (*b*) together	14·24	1·09	3·2	2·6
Exp. 2 (*a*) Standard	14·41	1·59	0·7	3·4
(*b*) Spray-dried milk	20·24	3·73	—	—
(*c*) Evaporated milk	18·06	2·55	—	—
(*a*) and (*b*) together	14·97	1·38	1·1	2·8
(*a*) and (*c*) together	14·76	1·29	0·9	2·8

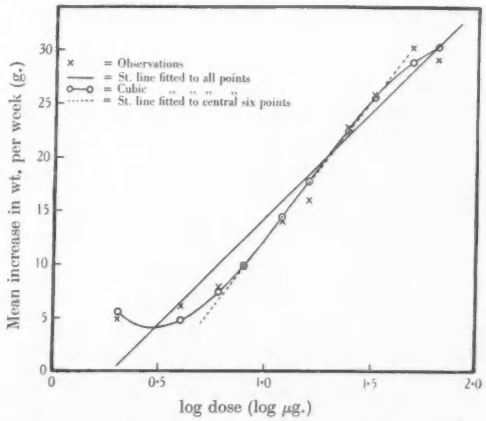


Fig. 2. Data of El Sadr *et al.* Relation between growth and log dose.

The usual test for linearity of regression [Fisher, 1936], however, shows a significant departure from linearity (the variance ratio is 10·4 with a 0·1 % point of only 3·8;

$$z = 1{\cdot}17, \quad 0{\cdot}1\,\%\ \text{pt.} = 0{\cdot}67).$$

It is clear that the straight line is too low for very low and rather high doses and too high elsewhere. The negative controls show that the response to low doses is largely non-specific while the response to the two highest doses suggests a slackening rate of increase. The data are satisfactorily graduated by the cubic curve

$$y = 14{\cdot}951 - 49{\cdot}239x + 64{\cdot}785x^2 - 18{\cdot}161x^3, \qquad \text{.....(2)}$$

the deviations from which are only such as might arise by chance (variance ratio = 1·1, 5% pt. = 2·2, z = 0·05, 5% pt. = 0·40).

In practice however it would be simpler and more satisfactory to use doses between the ranges 6 and 32 μg. The best fitting straight line to the responses within the range, that is to the central 6 doses, is

$$y = -13{\cdot}923 + 26{\cdot}357x. \qquad \text{.....(3)}$$

This gives a satisfactory fit, showing no significant departure from linearity (variance ratio = 2·5, 5% pt. = 2·6; z = 0·46, 5% pt. = 0·48).

The values yielded by equations (1), (2) and (3) are given in Table 2, while Table 6 gives the slopes of the two straight lines together with their standard errors (see also Fig. 2).

(2) *Data of Henry* et al. (*Tables 3, 4, 5 and 6*)

The data were obtained from two experiments. In the first, doses of 0·9 and 1·8 g. of spray-dried milk were compared with 5 doses of the standard. In the second, doses of 0·5 and 1·0 g. spray-dried milk and of 2·0 and 4·0 g. evaporated milk were compared with 4 doses of standard. The average responses are shown in Tables 3 and 4.

In each experiment the slopes of the straight lines relating response to log dose were calculated separately for the standard and for each milk. The pooled slopes were also calculated for standard and spray-dried milk and standard and evaporated milk, respectively. Linearity of regression was tested in each case and no significant departures from it were found. Table 6 gives the slopes and their standard errors together with the variance ratios and their corresponding 5% points for the linearity test. In the first experiment the slope for spray-dried milk is significantly higher than for the standard, the difference being 2·2 times its standard error, in the second experiment the difference is in the same direction but only 1·2 times its standard error and does not reach the significance level. None of the slopes in the second experiment differ significantly. The results of the three assays are given in Table 5.

(3) *Comparison of results*

The slope of the standard curve obtained from the data of El Sadr *et al.* is significantly steeper than the slopes for standard from the data of Henry *et al.* even if all the doses are included in the calculation of the former. This overall slope does not differ significantly from the slopes for milk.

The fact that significantly different slopes for the standard curve may occur in different laboratories emphasizes the necessity of using at least 2 doses of standard and 2 doses of the unknown preparation in routine testing.

REFERENCES

Ansbacher, Supplee & Bender (1936). *J. Nutrit.* **11**, 401.
Bessey (1938). *J. Nutrit.* **15**, 11.
—— & Wolbach (1939). *J. exp. Med.* **69**, 1.
Booher (1932). *J. biol. Chem.* **102**, 39.
—— Blodgett & Page (1934). *J. biol. Chem.* **107**, 599.
Bourquin & Sherman (1931). *J. Amer. chem. Soc.* **53**, 3501.
Carlsson & Sherman (1938). *J. Nutrit.* **15**, 57.
Cook, Clark & Light (1937). *Sci.* **85**, 503.
Copping (1936). *Biochem. J.* **30**, 849.
Day, Darby & Cosgrove (1938). *J. Nutrit.* **15**, 83.
—— —— & Langston (1937). *J. Nutrit.* **13**, 389.
Edgar, El Sadr & Macrae (1938). *Biochem. J.* **32**, 2200.
—— & Macrae (1937). *Biochem. J.* **31**, 886.
—— —— & Vivanco (1937). *Biochem. J.* **31**, 879.
El Sadr (1940). *J. Path. Bact.* **50**, 199, Proc.
—— Macrae, Work, Lythgoe & Todd (1939, 1). *Nature*, **144**, 73.
—— —— —— (1939, 2). *Biochem. J.* **33**, 611.
Emmerie (1938). *Z. Vitaminforsch.* **7**, 244.
Euler, Karrer, Adler & Malmberg (1934). *Helv. chim. Acta*, **17**, 1157.
Fisher (1936). *Statistical Methods for Research Workers*, 6th Edit., Sect. 44.
Folley, Ikin, Kon & Watson (1938). *Biochem. J.* **32**, 1988.
Ghalioungui & Hanna (1938). *J. Egypt. med. Ass.* **21**, 750.
Goldberger & Tanner (1925). *U.S. Pub. Hlth. Rep.* **40**, 54.
György (1935). *Biochem. J.* **29**, 741.
—— Von Klaveren, Kuhn & Wagner Jauregg (1934). *Hoppe-Seyl. Z.* **223**, 236.
Henry, Houston, Kon & Osborne (1939). *J. Dairy Res.* **10**, 272.
Hodson & Norris (1939). *J. biol. Chem.* **131**, 621.
Hughes (1939). *J. Nutrit.* **17**, 527.
Irwin (1937). *J. Roy. Statist. Soc. Suppl.* **4**, 4.
Jukes (1937). *J. Nutrit.* **14**, 223.
Karrer (1939). *Ergeb. vit. u. Hormonforsch.* **2**, 381.
Kinnersley, O'Brien, Peters & Reader (1933). *Biochem. J.* **27**, 225.
Kramer, Dickman, Hildreth, Kunerth & Riddell (1939). *J. Dairy Sci.* **22**, 753.
—— Gardner, Kunerth & Riddell (1938). *J. agric. Res.* **56**, 233.
Lindholm (1938). *Biochem. J.* **32**, 314.
Lunde, Kringstad & Olsen (1939). *Hoppe-Seyl. Z.* **260**, 141.
Macrae, Todd, Lythgoe, Work, Hind & El Sadr (1939). *Biochem. J.* **33**, 1681.
Margolis, Margolis & Smith (1939). *J. Nutrit.* **17**, 63.
Oden, Oden & Sebrell (1939). *U.S. Pub. Hlth. Rep.* **54**, 790.
Schmidt & Sydenstricker (1938). *J. Amer. med. Assoc.* **110**, 2065.
Sebrell & Butler (1938). *U.S. Pub. Hlth. Rep.* **53**, 2282.
—— & Onstott (1938). *U.S. Pub. Hlth. Rep.* **53**, 83.
Sherman & Langord (1938). *J. Amer. med. Ass.* **110**, 1278.
Snell & Strong (1939). *Industr. Engng Chem.* Anal. Ed. **11**, 346.
Street & Cowgill (1939). *Amer. J. Physiol.* **125**, 323.
Supplee, Bender & Jensen (1939). *Industr Engng Chem.* **11**, 495.
—— Flanigan, Hanford & Ansbacher (1936). *J. biol. Chem.* **113**, 787.
Vilter, Vilter & Spies (1939). *J. Amer. med. Ass.* **112**, 420.
Whitnah, Kunerth & Kramer (1938). *J. Dairy Sci.* **21**, 593.

73. STUDIES ON DIFFUSING FACTORS
4. THE MODE OF ACTION OF DIFFUSING FACTORS

By M. G. EVANS and J. MADINAVEITIA
From the Chemistry Department, University of Manchester

(*Received 24 February 1940*)

The assay methods which have so far been used to measure the potency of diffusing factor preparations depend on the ability of these factors to increase the area of skin penetrated by a suitable indicator on intracutaneous injection. This

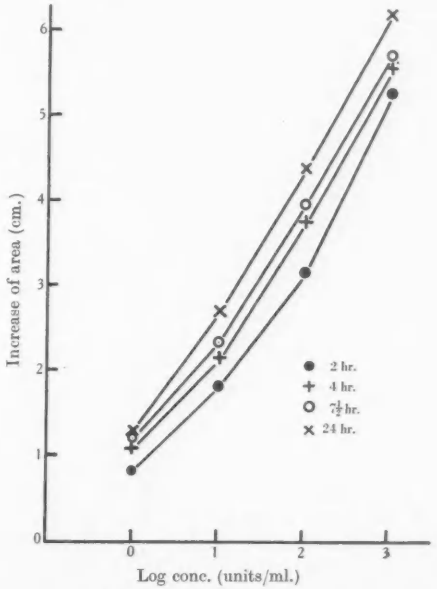


Fig. 1. Dose-response curve of testicular diffusing factor. Average of the results obtained in eighteen rabbits. Readings taken at different times.

increase is measured relative to a control injection without diffusing factor [Hofman & Duran-Reynals, 1931; McClean, 1930] or comparison is made with a standard preparation of diffusing factor [Madinaveitia, 1938]. In all these methods the margin of error is very large, and at best only consecutive tenfold dilutions can be differentiated with certainty. This very low accuracy is due rather to the remarkable flatness of the dose-response curve than to technical difficulties or individual variation in the experimental animals.

To establish the dose-response curve eighteen rabbits were used, four consecutive tenfold dilutions of the standard preparation of diffusing factor [Madinaveitia, 1938] being injected into the flanks of each animal. The solvent used was

an isotonic solution of haemoglobin and the experimental technique was that previously described [Madinaveitia, 1938]. The result is shown in Fig. 1, where, instead of representing the whole area penetrated by the haemoglobin as in previous papers, the increase in area relative to a control injection has been plotted; the figures obtained in this way are considered to represent the approximate net effect of the diffusing factor. Unless otherwise stated this method of representation is employed throughout this paper.

In Fig. 1 the average increase in the area of spread due to different concentrations of diffusing factor is plotted against the logarithm of the concentration. If the actual concentration were employed then with a scale of 1 cm. = 1 unit a strip of paper over 30 ft. in length would be necessary to represent the results. It seems at first sight astonishing that concentration should have so little effect on this manifestation of the action of diffusing factors. A possible explanation can be found if it be assumed that the active material present in the injected fluid is very rapidly eliminated or destroyed on coming into contact with the skin tissue. This assumption seems reasonable since it has been observed [Claude & Duran-Reynals, 1937] that intracutaneous injection of a concentrated solution of diffusing factor into the flank of a rabbit results after some time in the formation of a bleb of fluid in the abdominal skin of the animal, the injected fluid having fallen by gravity along the skin tissue and collected at its lowest point. The contents of such blebs were devoid of diffusing properties indicating that the tissues had destroyed or retained the active constituents. It is unlikely that they would be removed by the circulating blood since the action of diffusing factors is independent of circulation [Madinaveitia, 1939].

The work of McClean [1930] has shown that in the action of diffusing factors the spread takes place only in the true dermis. Working with coloured indicators he found that they penetrated neither the malphigian layer nor the epidermis.

The kinetics of the diffusing factor

The kinetics of the spreading of indicator solutions through the dermal layer might be governed by one or both of the following steps.

(*a*) A reaction between the diffusing factor molecules and the non-permeable wall of the dermal tissue.

(*b*) The diffusion of the molecules of "diffusing factor" into the layer of solution which has been depleted of diffusing factor molecules by the reaction.

Let us first consider the case in which this second step is much slower than the first and is therefore the process which is followed experimentally.

The law governing a diffusion process is

$$D\frac{\partial^2 c}{\partial x^2}=\frac{\partial c}{\partial t},$$

where c is the concentration of the diffusing substance, x the distance along the direction of diffusion, t the time and D the diffusion constant for the substance. If a is the thickness of the layers through which diffusion occurs, the differential equation for diffusion can be written (to within an accuracy of 0·4 %) in the integrated form

$$\frac{\bar{c}-c_0}{c_i-c_0}=1-\frac{8}{\pi^2}e^{-\frac{D\pi^2}{4a^2}t}, \text{ provided that } \frac{D\pi^2}{4a^2}t>0{\cdot}4$$

where $\bar{c}$ is the mean concentration of the diffusing substance in the layer of thickness a, c_0 the concentration of the diffusing substance in the layer at a time $t=0$ [Roughton, 1932] and c_i the concentration of the diffusing substance at the boundary of the layer; c_i is assumed to be constant.

In the experiments described here the diffusing substance, which may be the "diffusing factor" or the haemoglobin used as indicator, travels over a length of about 0·5 cm. in about 10 min. If then the concentration of the diffusing molecules is to remain constant over the whole layer the diffusion constant for the substance must be of the order of $D = 10^{-3}$–10^{-4}. This is a much higher diffusion constant than has been found for proteins, viz. $D \sim 10^{-7}$.

This diffusion expression can be used only if mechanical or thermal mixing effects are absent, and there is direct experimental evidence however that a slow diffusion process of molecules whose diffusion constants are comparable with those for proteins cannot be the rate-determining step.

If a layer of dermal tissue is treated with a solution containing the diffusing factor by injecting the solution and allowing it to spread to its full extent then it is found that a solution containing haemoglobin introduced into this pretreated layer spreads with great rapidity. Moreover, if a solution of diffusing factor containing haemoglobin as indicator is allowed to spread in the dermal layer the depth of colour is constant from the point of injection to the periphery of the spreading area at all times during the process. Since haemoglobin has a diffusion constant of 5×10^{-2} we should expect, if the diffusion occurs in an unmixed solution, a very marked change in the depth of colour, due to a steep gradient in concentration of haemoglobin, from the point of injection to the periphery of the area covered. That this is not the case would seem to indicate that mechanical or thermal mixing must occur to maintain a constant concentration of haemoglobin throughout the solution. Unless the diffusing factor molecules have a much larger diffusion constant than haemoglobin the same argument can be applied to the diffusing factor and we conclude that the liquid introduced into the dermal layer is mixed either by convection currents set up by the temperature difference between the two faces of the layer in which spreading occurs or by the mechanical effects of muscular action.

Reaction between the diffusing factor molecules and non-permeable wall

Let us now consider step (*a*), that is, reaction between the molecules of diffusing factor and the non-permeable wall of dermal tissue as the possible slow process.

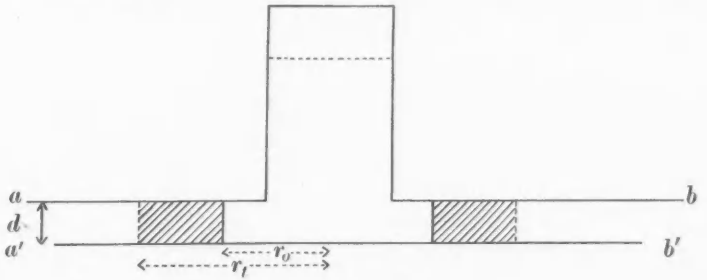


Fig. 2.

Fig. 2 gives a simplified model of the blister and the dermal layer which we shall use in this discussion.

The region between the two lines ab and $a'b'$ represents the dermal layer of thickness d in which spreading is occurring, r_0 represents the radius of the blister

at time $t=0$ and r_t that at time t. The shaded area represents the region over which spreading has occurred in the time t.

Let us postulate that a reaction occurs between the molecules of diffusing factor and the non-permeable wall and that permeability is conferred upon the wall when unit area of wall of unimolecular thickness takes up n molecules of diffusing factor. This means that the non-permeable wall is pushed back by a thickness dr, the thickness of a unimolecular layer, and the radius over which "free diffusion" through permeable tissue can occur is now r_0+dr.

The rate-determining step is the rate at which the wall can be made permeable by the reaction between the molecules of diffusing factor and the wall.

The experiments of Claude & Duran-Reynals indicate that the reaction between the diffusing factor molecules and the wall is practically irreversible and hence we need consider only the velocity of the forward reaction, that is the rate of disappearance of the diffusing factor molecules, in considering the overall velocity of the spreading process.

We can treat this reaction as one between the molecules of the solute and a "solid" surface of variable area. The rate $\frac{\partial x}{\partial t}$ at which the solute molecules react with the surface is proportional to their concentration and to the area of surface exposed at any time.

This is expressed by

$$-\frac{\partial x}{\partial t}=kc.A, \qquad \text{.....(1)}$$

where c is the concentration of the diffusing factor molecules and A the area of the non-permeable wall exposed to the solution at any time.

The quantities $\frac{\partial x}{\partial t}$, c and A have the following explicit values

$$c=\frac{1}{V}(x_0-\rho d\pi\,(r^2-r_0^2))$$

where ρ is the number of diffusing factor molecules taken up per unit volume of dermal wall, and x_0 the number of diffusing factor molecules initially present in the solution. The volume V may be written

$$V=V_0+\alpha d\pi\,(r^2-r_0^2)$$

where V_0 is the initial volume of liquid injected and α is the fractional volume of water in the volume of tissue through which spreading has occurred which contributes to the spreading liquid.

The area of wall exposed at any time to the solution is $A=2\pi rd$, where r is the radius of the area covered and d the thickness of the dermal layer.

The rate of change of the number of diffusing factor molecules with time is

$$\frac{\partial x}{\partial t}=-\frac{\partial}{\partial t}\rho d\pi\,(r^2-r_0^2)=-\rho d\pi\,2r\frac{\partial r}{\partial t}.$$

Equation (1) thus becomes

$$\frac{\partial r}{\partial t}=\frac{k\,[x_0-\rho d\pi\,(r^2-r_0^2)]}{\rho\,[V_0+\alpha d\pi\,(r^2-r_0^2)]},$$

thus giving an expression for the rate of change with time of the radius of the spreading area in terms of a reaction constant k, the number of spreading factor molecules initially present, the area covered and the initial volume. Equation (2), when integrated between the limits $r=r_0$ and $r=r$, gives

$$\frac{kt}{\rho}=\left[\tanh^{-1}r\sqrt{\frac{\rho d\pi}{(x_0+\rho d\pi r_0^2)}}\left\{\frac{V_0-\alpha d\pi r_0^2}{\sqrt{\rho d\pi\,(x_0+\rho d\pi r_0^2)}}+\frac{\alpha}{\rho}\sqrt{\frac{x_0+\rho d\pi r_0^2}{\rho d\pi}}\right\}-\frac{\alpha}{\rho}r\right]_{r=r_0}^{r=r}.$$

The volume of water contributed to the spreading solution from the volume of dermal tissue through which spreading has occurred is small compared with the volume of liquid injected. If α is so small that the volume $\alpha d\pi\ (r^2-r_0^2)$ can be neglected compared with V_0 the integrated expression reduces to

$$\frac{kt}{\rho}=\frac{V_0}{\sqrt{\rho d\pi\ (x_0+\rho d\pi r_0^2)}}\left\{\tanh^{-1} r\sqrt{\frac{\rho d\pi}{(x_0+\rho d\pi r_0^2)}}-\tanh^{-1} r_0\sqrt{\frac{\rho d\pi}{(x_0+\rho d\pi r_0^2)}}\right\}$$

writing $\pi r_0^2=A_0$ the area at time $t=0$ and $\pi r^2=A$ the area at any time t.

$$\frac{kt}{\rho}=\frac{V_0}{\sqrt{\rho d\pi\ (x_0+\rho dA_0)}}\left\{\tanh^{-1}\sqrt{\frac{\rho dA}{(x_0+\rho dA_0)}}-\tanh^{-1}\sqrt{\frac{\rho dA_0}{(x_0+\rho dA_0)}}\right\}$$

when the time reaches very large values $t\to\infty$, then the value of $\left(\frac{\rho dA_\infty}{x_0+\rho dA_0}\right)$ tends to unity, that is at $t=\infty$, then $x_0=\rho d\ (A_\infty-A_0)$ and the expression takes the simpler form

$$kt=\frac{V_0}{d\sqrt{\pi A_\infty}}\left\{\tanh^{-1}\sqrt{\frac{A}{A_\infty}}-\tanh^{-1}\sqrt{\frac{A_0}{A_\infty}}\right\}.$$

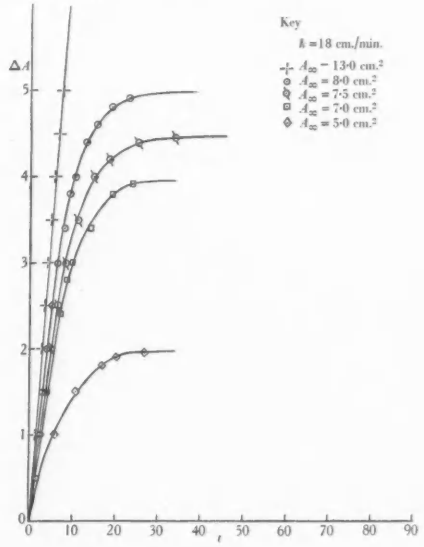


Fig. 3.

Figs. 3, 4 and 5 show the forms of the relation between area and time for various values of the constants in equation.

The important variables for this discussion are (i) the value of the velocity constant k, (ii) the number of diffusing factor molecules taken up per unit volume of dermal wall and (iii) the initial concentration of diffusing factor molecules in the solution.

Figs. 3 and 4 show the influence of change of x_0 and change of k respectively on the rate of spreading when ρ is large. Fig. 5 shows the influence of changes in x_0 and in k when ρ is small. One notices that the kallikrein behaviour is given

when k and ρ are small, whereas large values of these quantities lead to the diffusing factor behaviour. Fig. 6 shows both the experimental points for the increase in area under the influence of diffusing factor and kallikrein and in the

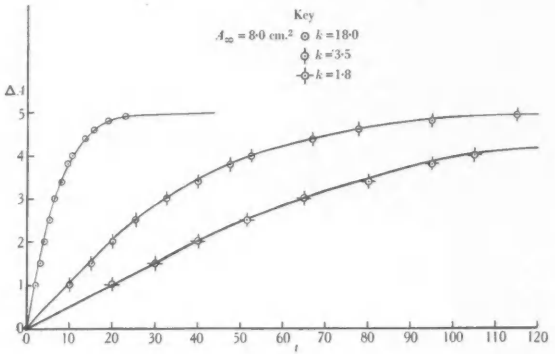


Fig. 4.

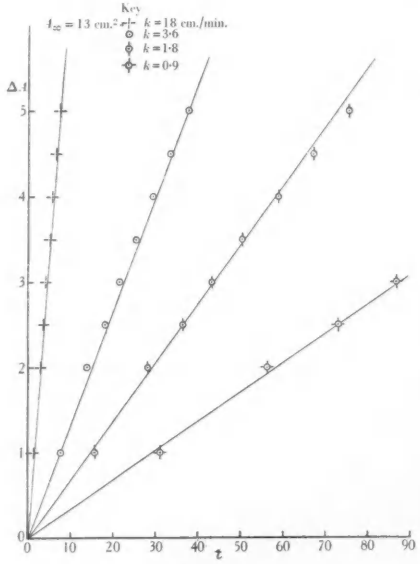


Fig. 5.

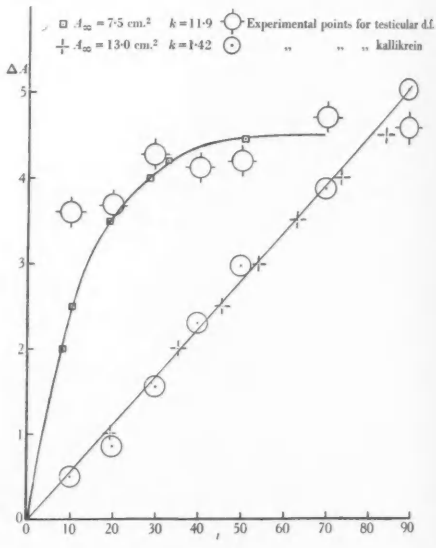


Fig. 6.

continuous lines values calculated from the equation using the magnitudes k for curve (i) 11·9 cm./min., k for curve (ii) 1·42 cm./min. and the ratio of

$$\frac{\rho \text{ curve (i)}}{\rho \text{ curve (ii)}} = \frac{1{\cdot}8}{1}.$$

The expression developed here while accounting satisfactorily for the rate of spreading does not account for the small effect which change of concentration of the diffusing factor has upon the area attained after some hours. If the mechanism outlined here were to hold until equilibrium had been achieved we should expect the final area to increase linearly with the concentration of diffusing factor molecules.

This is not the case as is seen from Fig. 1. The following may be advanced as a possible explanation. As well as the rapid reaction occurring between the diffusing factor molecules and the non-permeable dermal layer (which reaction determines the velocity in the early stages of the process) there is also a much slower reaction which can occur between the diffusing factor molecules and "wall" which has already been made permeable.

The nature of such a reaction is not yet clear, but if it were an adsorption process then in the later stages of the diffusion process, when the number of molecules of the dermal layer which have been rendered permeable is very large, the adsorption would lead to a more rapid loss of diffusion factor molecules. Consequently the final area achieved would be related to the initial concentration by

$$x_0 = \rho d\,(A_\infty - A_0) + f\,(A_\infty - A_0),$$

where the first term gives the amount of diffusion factor which has been removed by making the wall permeable and the second term that which has been removed by reaction with the substrate after it has been made permeable.

If the slower process is an adsorption process it may obey a law of the type $f\,(A_\infty - A_0) = k_2 x_0^{1/n}\,(A_\infty - A_0)\,d$ where k_2 and n are the constants of the adsorption isotherm when the increase in area at infinite time is given by

$$(A_\infty - A_0) = \frac{x_0}{\rho d + k_2 x_0^{1/n}}.$$

Fig. 7 shows the type of curve resulting from this relationship.

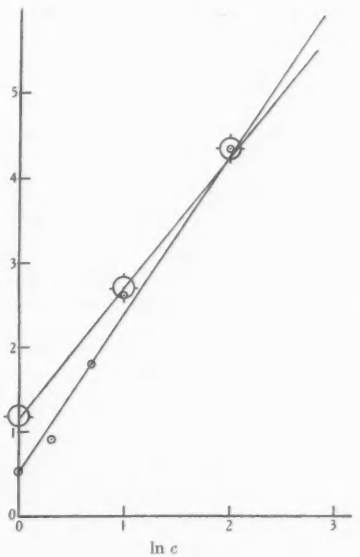


Fig. 7.

McClean *et al.* [1933; 1934] have studied the influence of testicular diffusing factor on the rate of absorption of diphtheria antitoxin when administered subcutaneously. They injected into guinea-pigs mixtures containing a fixed amount of antitoxin and varying quantities of diffusing factor, and observed that an increase in the concentration of the latter caused an increase in the number of units of antitoxin circulating in the blood of the animals. Since the testicular diffusing factor has no effect on the blood circulation [Madinaveitia, 1939] this increased passage of antitoxin into the circulation probably indicates that the diffusing factors increase the permeability of the subcutaneous layer just as they increase that of the dermis. This would cause the injected fluid to spread over a larger area and so increase the number

of capillaries coming in contact with it and capable of conveying the antitoxin. The type of dose-response curve obtained on plotting the experimental results of McClean *et al.* [1934] is similar to the dose-response curve obtained with coloured indicators in the skin of rabbits (Fig. 5).

SUMMARY

The mode of action of diffusing factors is discussed and a possible theoretical explanation advanced.

The authors' thanks are due to Prof. A. R. Todd for his helpful interest.

REFERENCES

Claude & Duran-Reynals (1937). *J. exp. Med.* **65**, 661.
Hofman & Duran-Reynals (1931). *J. exp. Med.* **53**, 387.
Madinaveitia (1938). *Biochem. J.* **32**, 1045.
—— (1939). *Biochem. J.* **33**, 347.
McClean (1930). *J. Path. Bact.* **33**, 1045.
—— & Morgan (1933). *J. Path. Bact.* **36**, 193.
—— —— & Favilli (1934). *J. Path. Bact.* **38**, 253.
Roughton (1932). *Proc. roy. Soc.* B, **111**, 1.

74. STUDIES ON DIFFUSING FACTORS

5. SEPARATION OF THE DIFFUSING FACTOR FROM THE PROTEOLYTIC ENZYMES PRESENT IN CULTURE FILTRATES OF *CLOSTRIDIUM WELCHII*

By J. MADINAVEITIA

From the Chemistry Department, University of Manchester

(*Received 29 February 1940*)

INDIRECT evidence has recently been advanced [Madinaveitia, 1939] to show that the diffusing factor is not a proteolytic enzyme; since, under the experimental conditions used, testicular extracts did not hydrolyse gelatin it seemed almost certain that if diffusing factors from different sources are similar in their physiological action, their effects could not be due to proteolysis. A more conclusive proof is the separation of proteolytic and diffusing activities in culture filtrates of *Clostridium Welchii* which has now been accomplished. Despite the qualitative nature of the assay method for diffusing activity and its very large margin of error it was possible to demonstrate this separation in culture filtrates fractionated by various methods.

A dialysed *Cl. Welchii* filtrate adjusted to *p*H 4·0 deposits on standing a precipitate which contains most of the proteolytic activity of the starting material but is devoid of diffusing properties. Separation can also be effected by fractional adsorption on alumina C-γ or by fractional elution of kaolin or alumina adsorbates. It is evident from the results that the diffusing factor of *Cl. Welchii* is quite distinct from the gelatin-splitting enzymes produced by this organism.

Experimental

Culture filtrates of *Cl. Welchii* were concentrated *in vacuo* (23°) to 1/10 of their volume and dialysed for two days against running tap water. The dialysed solutions were concentrated *in vacuo* (23°) to the original volume of culture filtrate.

For estimation of the proteolytic activity of the different fractions 1 ml. of enzyme solution was incubated with 9 ml. 5% gelatin in *M*/15 phosphate buffer *p*H 7·0 containing 0·05% cysteine hydrochloride. The increase of carboxyl groups was estimated in 2 ml. samples after 15–24 hr. incubation at 38° by the method of Willstätter & Waldschmidt-Leitz [1921].

The diffusing activities of the different fractions were compared by the recently described modification [Madinaveitia, 1939] of the original method [Madinaveitia, 1938]. The figures given represent the average of double determinations in at least two rabbits.

Concentration of the proteolytic activity

To portions of 10 ml. of the dialysed filtrate 1 ml. of *M* acetate buffer of varying *p*H was added and each portion adsorbed with 0·5 g. kaolin (B.D.H. acid-washed). After 1 hr. the adsorbates were centrifuged off and the proteolytic activity of each supernatant estimated.

Table 1

	Original solution	Supernatants			
		*p*H 3	*p*H 4	*p*H 5	*p*H 6
Increase in ml. *M*/20 KOH	1·30	0·15	0·20	0·60	1·30

Further adsorptions were always carried out at *p*H 4·0 because of the danger of destroying the diffusing factor at a lower *p*H.

Different eluents were tried. 10 ml. lots of a dialysed culture filtrate adjusted to *p*H 4·0 were adsorbed with 1 g. of kaolin. After separation of the supernatants, the adsorbates were eluted with 10 ml. of the eluent by shaking for 10 min. The proteolytic activities of the eluates were determined.

Table 2

	Increase in ml. *M*/20 KOH
Original solution	1·25
Eluent:	
M/100 acetic acid	0·20
M/15 KH_2PO_4	0·40
Water	0·40
M/15 Na_2HPO_4	0·55
1% Na_2CO_3	0·40
M/100 NaOH	1·00

In order to determine the minimum amount of kaolin required for adsorption of the proteolytic activity 10 ml. portions of the above-mentioned buffered dialysed culture filtrate were adsorbed with varying amounts of kaolin, eluted in each case with 10 ml. *M*/100 NaOH, and the proteolytic activity of each of the eluates estimated.

Table 3

	Increase in ml. *M*/20 KOH
Original solution	1·50
g. kaolin used:	
0·05	0·65
0·1	1·30
0·3	1·35
0·5	1·40
0·7	1·45

Separation of the proteolytic enzymes from the diffusing factor

A. *By acid precipitation.* To 5 ml. of a dialysed culture filtrate 0·5 ml. *M* acetate buffer *p*H 4·0 was added and the mixture allowed to stand overnight at room temperature. The precipitate was centrifuged off and washed with 5 ml. *M*/10 acetate buffer *p*H 4·0, again centrifuged and dissolved in 5 ml. *M*/10 borate buffer *p*H 9·2.

Table 4

	Increase in ml. *M*/20 KOH	Increase in area of spread cm.^{2}
Original solution	1·15	5·10
Acetic acid supernatant	0·40	4·95
Washings	0·00	0·87
Precipitate	0·70	0·65

B. *By fractional adsorption.* Both the diffusing factor and the proteolytic enzymes can be adsorbed on alumina C-γ, but the amount of adsorbent required for adsorption of the proteolytic enzymes is much smaller than for the diffusing factor. To 5 ml. of a dialysed culture filtrate 0·5 ml. *M* acetate buffer *p*H 4·0 was added and the mixture shaken with 0·1 ml. alumina C-γ (21 mg. Al_2O_3/ml.). After 10 min. the adsorbate was centrifuged off, washed with 5 ml. *M*/10 acetate buffer *p*H 4·0 and eluted with 5 ml. *M*/10 borate buffer *p*H 9·2.

Table 5

	Increase in ml. *M*/20 KOH	Increase in area of spread cm.^{2}
Original solution	1·85	5·69
Supernatant	0·10	4·93
Washings	0·00	0·66
Eluate	1·60	1·91

C. *By selective elution.* From adsorbates of *Cl. Welchii* culture filtrates the diffusing factor is eluted at a much lower *p*H than the proteolytic enzymes.

10 ml. of dialysed culture filtrate mixed with 1 ml. *M* acetate buffer *p*H 4·0 were shaken with 2 ml. alumina C-γ. After 10 min. the precipitate was centrifuged and eluted with 10 ml. *M*/10 Na_2HPO_4.

Table 6

	Increase in ml. *M*/20 KOH	Increase in area of spread cm.^{2}
Original solution	1·35	4·26
Supernatant	0·05	1·63
Eluate	0·15	4·78

M/100 NaOH elutes from kaolin adsorbates both the diffusing factor and the proteases. 10 ml. of a dialysed culture filtrate buffered with 1 ml. *M* acetate buffer *p*H 4·0 were adsorbed by shaking with 0·7 g. kaolin. After 10 min. the adsorbate was centrifuged off and eluted with 10 ml. *M*/100 NaOH.

Table 7

	Increase in ml. *M*/20 KOH	Increase in area of spread cm.^{2}
Original solution	1·05	3·19
Supernatant	0·00	0·12
Eluate	1·10	3·62

0·4% ammonia has the same eluting properties as *M*/100 NaOH. 1% pyridine, on the other hand, elutes only the diffusing factor.

Table 8

	Increase in ml. *M*/20 KOH	Increase in area of spread cm.^{2}
Original solution	0·70	4·62
Supernatant	0·00	0·30
Ammonia eluate	0·50	3·92
Pyridine eluate	0·05	4·22

It was necessary to prove that pyridine has no great effect on the action of the proteolytic enzyme. For this purpose 1 ml. of dialysed culture filtrate was incubated with 1 ml. 2% pyridine for 1 hr. at 38°. The proteolytic activity of

this incubated solution was compared with that of a 1/1 dilution of the original culture filtrate. Both solutions gave approximately the same increase of carboxyl groups.

SUMMARY

Various methods are described whereby the proteases present in *Cl. Welchii* filtrates can be separated from the diffusing factor.

The author's thanks are due to Prof. A. R. Todd for his continued interest and advice. He is also grateful to Dr D. McClean of the Lister Institute (Elstree) for supplies of *Cl. Welchii* filtrates and to Imperial Chemical Industries, Ltd., for grants.

REFERENCES

Madinaveitia (1938). *Biochem. J.* **32**, 1806.
—— (1939). *Biochem. J.* **33**, 347.
Willstätter & Waldschmidt-Leitz (1921). *Ber. dtsch. chem. Ges.* **561**, 2988.

75. STUDIES ON DIFFUSING FACTORS

6. THE ACTION OF TESTICULAR EXTRACTS ON THE VISCOSITY OF VITREOUS HUMOUR PREPARATIONS

By J. MADINAVEITIA and T. H. H. QUIBELL

From the Chemistry Department, University of Manchester

(*Received 29 February 1940*)

Chain & Duthie [1939] have recently shown that testicular extracts contain an enzyme capable of hydrolysing the mucins of synovial fluid and vitreous humour. Mucinases of a similar type have been obtained from pneumococci [Dubos, 1937; Meyer *et al.* 1937] and from haemolytic streptococci [Meyer, 1938]. Since the first visible effect of the testicular enzyme on the mucins examined was to reduce their viscosity and since there is histological evidence for the existence of a mucin-like interfibrillar substance in skin [Bensley, 1934; Sylven, 1938], Chain & Duthie suggested that the diffusing factor and the mucinase in testicular extracts might be identical. This view is in accordance with their similarity in properties and their common occurrence in testicle and some bacterial filtrates. If we are to consider the characteristic effect produced by diffusing factors as being due to enzyme action in the skin it is reasonable to assume that the increased spread of injected fluids is due to the ease with which they diffuse through tissues rendered less viscous by the action of the enzyme. Apart from mucinases other enzymes which might affect the viscosity of skin constituents are the proteases which can liquefy gelatin; it has, however, been shown [Madinaveitia, 1940] that in *Cl. Welchii* filtrates at least, the proteases can be separated from the diffusing factor.

It is not yet known whether testicular extracts contain a single mucolytic enzyme or a polysaccharase and an oligosaccharase. In the latter case the increase in the reducing properties of the substrate brought about by the extract would be a measure of the combined effect of the enzymes; the decrease in viscosity would be a measure of the polysaccharase activity only and, incidentally, a measure of the diffusing factor activity should it prove that the diffusing factor is indeed identical with the mucinase. The decrease in viscosity of a mucin by the action of a mucinase is a catalytic process and the disappearance of the viscous material should obey the laws of a unimolecular reaction. It has been experimentally confirmed, using testicular mucinase, that the reaction constant is independent of the substrate concentration (Table 1) and that the half-life time of a given amount of viscous substrate is inversely proportional to the concentration of the enzyme with which it reacts (Table 2). The substrate used was an acetone powder of bovine vitreous humour. The viscosity of its aqueous solutions is greatly affected by salts. Addition of small amounts of salts causes the viscosity to fall to a minimum; on further addition the viscosity again rises, the increase being parallel to that produced by adding salts to water alone. Based on these observations a viscosimetric method has been developed for the determination of mucinases. A 1% solution of vitreous humour in a buffered *M* NaCl solution is allowed to react with a measured amount of enzyme solution. The time required to reduce the specific viscosity of the reacting mixture to half its original value is inversely proportional to the amount of enzyme used.

Determination of the optimum *p*H for the action of testicular mucinase indicates that it is about *p*H 4·7. This is somewhat lower than the optimum found by Meyer *et al.* [1937] for the mucinase of pneumococcus; the difference might well be due to differences in the substrate or in the technique employed for estimating the enzyme activity. Since the enzyme has maximum stability in the neighbourhood of *p*H 4·7 estimation of mucinase potency is always carried out in a citrate buffer at this *p*H.

A variety of solvents have been used for extracting the enzyme from dried testicle powder. *M*/10 acetic acid is much better for this purpose than water; autolysis of the testicle powder offers no advantage over direct acetic acid extraction. From an acetic acid extract (*p*H 4·2) the mucinase precipitates at between 30 and 70 % of saturation with $(NH_4)_2SO_4$. From the solution obtained by redissolving this precipitate in water the active constituents are very difficult to adsorb on kaolin and, once adsorbed, they are almost impossible to elute. If, on the other hand, the solution be dialysed before treatment with kaolin adsorption is easily effected and the mucinase is readily eluted by a phosphate buffer solution. From the eluate obtained in this way the enzyme can be readily re-adsorbed on alumina C-γ and again eluted with phosphate buffer solution. Purified in this way the enzyme solutions show diffusing activity of an intensity similar to that of a crude testicular extract with the same mucinase activity.

It is so far impossible to state whether the mucinase and diffusing factor are identical although the point is clearly of great importance. Whether rigid identification will be possible must await further work.

Experimental

Substrate. The vitreous humour of 120 cattle eyes (1·2 l.) was thoroughly stirred and then filtered with suction through three layers of muslin. 1·1 l. of filtrate were run in a small jet into 10 l. of mechanically stirred acetone cooled to 0°. After standing at room temperature overnight the asbestos-like precipitate was collected by filtration, washed with a further litre of acetone and subsequently with 1 l. ether. During the whole process the precipitate was kept covered with solvent. After washing with ether the precipitate was dried and powdered. 4·5 g. of a very light white powder were obtained. This preparation is not completely soluble in water or in NaCl solutions. It is impossible to clarify the solutions by filtration through paper since the viscous material is retained by the filter; centrifuging on the other hand yields clear viscous solutions. The concentration of substrate will be given as percentage of the acetone powder in the clarified solution.

The viscosity of solutions of the substrate is an easily reproducible figure. Weighed amounts of substrate were extracted in a mortar with 100 times the weight of water. The water was added in small portions, no further addition being made until the contents of the mortar were homogeneous. When all the solvent had been added the solutions were centrifuged and the supernatants decanted through glass wool. Four different samples of solutions thus prepared required 336·5, 338·8, 330·8 and 336·2 sec. respectively to flow through an Ostwald viscosimeter of about 4·5 ml. capacity. Water tested under the same conditions required 112·5 sec.

Effect of NaCl on the viscosity of the substrate. 5 ml. samples of a 0·5 % solution of substrate in distilled water were mixed with 5 ml. of NaCl solutions of different concentrations, the concentration of substrate being thus reduced to 0·25 %. Another series in which the final concentration of substrate was 0·8 % was

prepared in a similar way. The flow time of each of the solutions at 25° is recorded in Fig. 1 as well as the flow time of NaCl solutions of different strength. Between 3 and 7 % the concentration of NaCl has very little effect on the flow time.

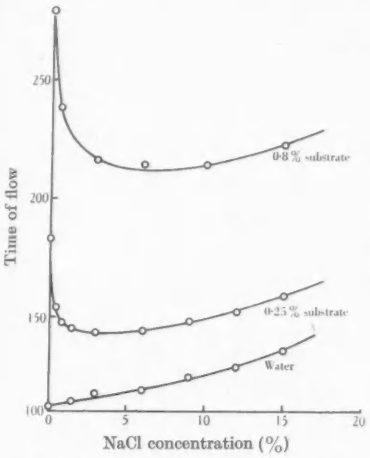


Fig. 1. Effect of NaCl on the viscosity of the substrate.

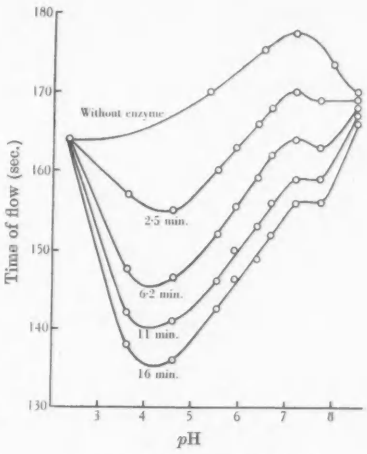


Fig. 2. Effect of *p*H on mucinase activity.

Variation of the viscosity of the substrate with p*H and its influence on the action of testicular extract.* To 4 ml. samples of 1 % substrate in *M* NaCl 1 ml. *M*/10 buffer solution was added. The great buffering capacity of the substrate between *p*H 6 and 7 makes the *p*H of the resulting mixtures different from that of the buffer used. The flow time of these solutions at 25° is recorded in Fig. 2.

The testicular extract used was prepared by grinding 1 g. dry testicle powder with 10 ml. water in a mortar. The turbid supernatant obtained by centrifuging was diluted with 9 vol. of water. To 4 ml. of the 1 % substrate solution above mentioned 1 ml. *M*/10 buffer and 1 ml. diluted testicular extract were added. In Fig. 2 the flow times at different intervals are plotted. The whole experiment was carried out at 25° with the precautions and corrections described elsewhere (p. 628). It will be seen that the enzyme action is most rapid at about *p*H 4·5–4·7.

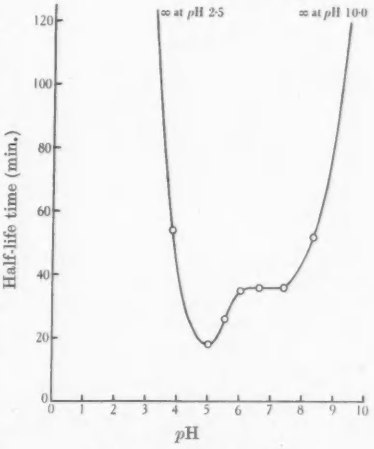


Fig. 3. Stability of mucinase.

Stability of mucinase at different p*H.* The centrifuged extract of 1 g. dry testicle powder with 10 ml. water was adjusted to *p*H 4·2 with *M* acetic acid.

1 ml. portions of the clear solution were made up to 10 ml. with suitable $M/50$ buffer solutions. After 18 hr. at 38° (thermostat) the mucinase content of the different samples was estimated (Fig. 3).

Effect of the concentration of substrate on the rate of reaction. 4 vol. of a 4% substrate solution in M NaCl and 1 vol. M Na citrate buffer pH 4·7 were mixed. Consecutive twofold dilutions of this solution up to 1/16 were made, using as a diluent a mixture of 4 vol. M NaCl and 1 vol. M Na citrate buffer pH 4·7. The source of enzyme used was a centrifuged extract of 1 g. dry testicle powder with 10 ml. M acetic acid diluted with 10 vol. water. 4 ml. portions of the different dilutions of substrate were allowed to react with 1 ml. enzyme solution at 25°. The viscosity of the reacting mixture was measured at different intervals. The flow time was plotted against reaction time and the initial flow time of the enzyme-substrate mixture (f_0) extrapolated. When the action of the enzyme on the substrate had proceeded long enough the flow time was reduced to that of the solvent in which the reaction takes place ($f_\infty = 127$ sec.). $\frac{1}{2}(f_0+f_\infty)$ has been taken as the flow time when half of the substrate has been rendered non-viscous. The time at which the flow time reaches this value is the half-life time of the substrate (τ). The reaction constant (k) was calculated avoiding the use of the initial flow time (f_0) which is an assumed value. Table 1 records the

Table 1

c	Concentration of substrate (in arbitrary units)	4	2	1	0·5	0·25
f_0	Initial flow time (in sec.) (extrapolated)	532	290	192	158	142
$\frac{1}{2}(f_0+f_\infty)$	Flow time at half concentration of viscous material (in sec.)	360	209	160	142	134·5
τ	Half-life time of the viscous material (in min.)	29·5	29	30	31	29
$k \cdot 10^{-2}$ min.$^{-1}$	Reaction constant $\times 10^{-2}$ min.$^{-1}$	9·25	9·44	9·92	8·90	—

results thus obtained. The reaction constant and the half-life time of the viscous substrate are tolerably constant, particularly at the higher concentrations of substrate where the experimental errors are smallest.

Effect of the enzyme concentration on the rate of reaction. 45 ml. 1% substrate solution in M NaCl were mixed with 15 ml. M acetate buffer pH 4·6. The enzyme used was the centrifuged extract of 1 g. dry testicle powder with 10 ml. of water. 1 ml. portions of different dilutions of enzyme were allowed to react with 4 ml. of the buffered substrate and the flow time determined at different intervals. The half-life time of the viscous substrate was estimated as above indicated. The results are recorded in Table 2. It will be observed that the concentration of enzyme is inversely proportional to the half-life time since the product is constant.

Table 2

Concentration of enzyme	50	33	20	10	3·3
Half-life time (min.)	8	14	20	42	130
$c \times \tau$	400	455	400	420	425

Method used for the comparison of the relative mucinase contents of testicular extracts. The half-life time of the substrate being inversely proportional to the concentration of enzyme its magnitude gives a convenient measure for the mucinase content of a given preparation. It has been shown that the half-life time is independent of the concentration of substrate. However, in order to

avoid experimental errors, the solutions of substrate used had a flow time between $\frac{1}{3}$ and $\frac{1}{15}$ of the half-life time. Their flow time was about 1·3–1·7 times that of the solvent. 5 ml. of a 1% solution in *M* NaCl of the acetone powder of bovine vitreous humour mixed with 1/5 vol. of *M* Na citrate buffer and 1/5 vol. of water had a flow time of about 200 sec. (f_0) in an Ostwald viscosimeter of about 4·5 ml. capacity in which the flow time of water measured under the same conditions was 112 sec. and the flow time of a mixture of 4 parts of *M* NaCl, 1 part of *M* Na citrate buffer and 1 part of water was 127 sec. (f_∞). All measurements were made in a thermostat at $25 \pm 0{\cdot}05°$ and the total volume of fluid placed in the viscosimeter was 6 ml. The value of f_0 was determined for each batch of the substrate solution.

5 ml. of a mixture of 4 vol. of a 1% solution of substrate in *M* NaCl and 1 vol. *M* Na citrate buffer were placed in the viscosimeter which was kept in the thermostat at 25°. After the temperature had equilibrated (about 5 min.), 1 ml. of the enzyme solution under test (at 25°) was added. A stop watch (*A*) was started, the contents of the viscosimeter thoroughly mixed and blown into the bulb. When the meniscus reached the top mark of the viscosimeter a second stop watch (*B*) was started and the time in stop watch *A* read ± 5 sec. (t). When the meniscus reached the lower mark stop watch *B* was stopped, the time read (f_t), the fluid again made to fill the bulb and the procedure repeated; $t + \frac{1}{2}f_t$ is considered to be the time required for the reacting mixture to have the flow time f_t. $t + \frac{1}{2}f_t$ was plotted against f_t and the point at which the curve cuts the line $\frac{1}{2}(f_0 + f_\infty)$ was taken as the half-life time of the viscous substrate or as the inverse of the concentration. If the half-life time of the substrate is between 10 and 40 min. it can be determined with an error of less than ± 1 min., the error of the method being therefore about ± 5%.

Extraction of the mucinase from dried testicle powder. 1 g. of the powder mixed with 2 g. of sand was ground in a mortar for 10 min. with 10 ml. of water. After centrifuging, 5 ml. supernatant were obtained. The residue was ground with a further 10 ml. water and centrifuged again (7·5 ml. supernatant). The residue was extracted a third time with 10 ml. water (8·5 ml. supernatant). The supernatants were precipitated by adjusting them to *p*H 4·1 with *M* acetic acid and the precipitates removed by centrifuging. The third supernatant had 13% of the mucinase content of the first one. Thus most of the mucinase was removed in the first extraction. Addition of acetic acid (*p*H 4·1) to the aqueous extracts produces a copious precipitate of inactive material. Extraction of 1 g. of dried testicle powder with 10 ml. *M*/10 acetic acid under the conditions described above yielded a solution having 1·5 times more mucinase per ml. than when water was used.

1 g. of testicle powder ground with 2 g. of sand and 10 ml. *M*/10 Na_2HPO_4 was allowed to autolyse at 38° for 24 hr. with 1 ml. toluene. The precipitate was centrifuged and the supernatant precipitated with acetic acid. The clear solution finally obtained had 1·7 times the mucinase content of a non-autolysed aqueous extract. Autolysis has therefore no great advantage over direct acetic acid extraction.

Ammonium sulphate fractionation. 50 g. dried testicle powder, 100 g. sand and 1 l. *M*/10 acetic acid were ground in a mortar for 1 hr. In 750 ml. of the clear extract (*p*H 4·2) 166 g. $(NH_4)_2SO_4$ were dissolved (30% of saturation). The inactive precipitate was removed and to the filtrate a further amount of 239 g. $(NH_4)_2SO_4$ was added, the resulting solution being 70% saturated with $(NH_4)_2SO_4$ (20°). The precipitate was collected, washed with 50 ml. 70% saturated $(NH_4)_2SO_4$ and dissolved in 250 ml. of water. In this final solution 80% of the mucinase

present in the acetic acid extract was found. The solution (*p*H 4·5) loses about 15 % of its mucinase content per day when kept at +5°.

Adsorption and elution of the mucinase. Two 10 ml. portions of the redissolved $(NH_4)_2SO_4$ precipitate (*p*H 4·3) were adsorbed with 0·5 and 2 g. kaolin respectively. 30 % of the mucinase was adsorbed by 2 g. kaolin but none by 0·5 g. 180 ml. of redissolved $(NH_4)_2SO_4$ precipitate were shaken with 49 g. kaolin which adsorbed 30 % of the nitrogen but none of the mucinase present. 10 ml. samples of the solution thus purified were adsorbed with 2 g. kaolin which removed more than 90 % of the mucinase and 61 % of the nitrogen. No mucinase was eluted from the adsorbates by shaking them with 10 ml. portions of *M*/10 acetic acid, water, *M*/15 KH_2PO_4, 2 % pyridine or 0·8 % NH_3. 10 ml. *M*/10 borate buffer (*p*H 9·2) or 10 ml. *M*/5 Na_2HPO_4 (*p*H 8·3) only elute about 10 % of the adsorbed enzyme.

100 ml. of the redissolved $(NH_4)_2SO_4$ precipitate were dialysed in a cellophane bag against running tap water during 36 hr. the volume increasing to 250 ml. After removing the inactive precipitate 10 ml. samples of the dialysed solution (*p*H 5·6) were adsorbed by shaking with different amounts of kaolin.

g. kaolin	0·2	0·5	0·7	1·0	1·5	2·0
% mucinase adsorbed	25	85	96·5	—	—	—
% N adsorbed	14	35	—	57	77·5	87·5

10 ml. samples of dialysed $(NH_4)_2SO_4$ precipitate were adsorbed with 0·5 g. kaolin. The adsorbates were eluted with 10 ml. *M*/10 phosphate buffers of different *p*H.

*p*H of buffer	4·38	7·37	6·97	6·68
% of adsorbed mucinase eluted	45	45	34·5	16

100 ml. of dialysed $(NH_4)_2SO_4$ precipitate were shaken with 5 g. kaolin for 30 min. The adsorbate was eluted with two 50 ml. lots of *M*/10 phosphate buffer *p*H 7·3. The combined eluates contained 55 % of the adsorbed mucinase. Dialysis in cellophane bag against running tap water for 18 hr. yielded 200 ml. of solution. Two 10 ml. portions of the dialysed kaolin eluate were adsorbed with 0·2 and 0·5 ml. alumina C-γ (19 mg. Al_2O_3/ml.) the volume being made up to 15 ml. in each case. The amounts of mucinase adsorbed were 15 and 77 % of that of the original solution. Elution with 15 ml. *M*/30 phosphate buffer *p*H 7·3 of the adsorbate with 0·5 ml. alumina recovered 85 % of the adsorbed mucinase.

The diffusing factor content of the alumina eluate was compared with that of a 1/20 dilution of an extract of 1 g. dry testicle powder with 10 ml. *M*/10 acetic acid. Double determinations were made in two rabbits by the modified technique recently described [Madinaveitia, 1939].

	Mucinase	Increase of the area of spread cm.²
Crude testicular extract	1·35	5·54
Purified mucinase	1·00	5·10

Summary

A viscosimetric method is described whereby testicular mucinase may be estimated with an error considerably less than 5 %. In the preliminary concentration of the enzyme from testicle powder mucinase activity seems to run roughly parallel with diffusing activity. No definite evidence has been obtained that the mucinase and diffusing factor are identical.

The authors' thanks are due to Prof. A. R. Todd for his continued interest and advice. They are also grateful to Imperial Chemical Industries, Ltd., for grants.

REFERENCES

Bensley (1934). *Anat. Rec.* **60**, 95.
Chain & Duthie (1939). *Nature, Lond.*, **144**, 977.
Dubos (1937). *J. exp. Med.* **65**, 347.
Madinaveitia (1939). *Biochem. J.* **33**, 347.
—— (1940). *Biochem. J.* **34**, 621.
Meyer (1938). *Cold Spring Harbor Symposia on Quantitative Biology*, **6**, 91.
—— Dubos & Smyth (1937). *J. biol. Chem.* **118**, 491.
Sylven (1938). *Virchows Arch.* **303**, 280.

76. THE EFFECT OF ADMINISTRATION OF AN ACETATE ON THE DETOXICATION AND THERAPEUTIC ACTIVITY OF SULPHANILAMIDE. II

By G. V. JAMES[1]

From the Bernhard Baron Research Laboratories, Queen Charlotte's Hospital, Hammersmith, W. 6

(*Received 23 January 1940*)

In a previous paper [James, 1939] the question of the exact manner by which sulphanilamide and acetylsulphanilamide exerted their toxic action was raised. It was suggested that the acetyl derivative had a direct specific toxic action whilst sulphanilamide might kill by a sudden withdrawal of acetate precursors from the body. The following experiments were undertaken to throw further light on the problem. The work was extended to cover sulphapyridine.

Fuller [1937] was the first to suggest the presence of a conjugated sulphanilamide in urine and Marshall *et al.* [1937] independently isolated this substance from human and rabbit urine. They obtained chemical constants for it and mentioned the previous work of Fuller. In further experiments Marshall *et al.* [1938] demonstrated its increased toxicity as compared with sulphanilamide.

Southworth [1937] was the first to observe a fall in the CO_2 combining power of the blood of patients receiving sulphanilamide; Hartmann *et al.* [1938] therefore suggested the administration of sodium lactate and Lucas & Mitchell [1939] $NaHCO_3$ to counteract this fall.

Lactate and bicarbonate would both raise the CO_2 capacity but the lactate has the advantage of being an acetyl precursor. During this fall in CO_2 capacity the production of an alkaline urine has been noted [Strauss & Southworth, 1938; Beckmann, 1938]; this is of interest as sulphanilamide is more bactericidal in an alkaline urine than in an acid one [Helmholz & Osterberg, 1937], acetylsulphanilamide being inactive at any reaction.

A fall of CO_2 capacity was also noted in man and dogs by Ocklerblad & Carlson [1939] when giving acetylsulphanilamide. So far these or similar changes have not been reported when sulphanilamidopyridine has been given, but Antopol & Robinson [1939] observed uroliths of acetylsulphanilamidopyridine in kidneys of rats, rabbits and monkeys which had received the base; Gross *et al.* [1939] analysed actual concretions from rat's kidneys and found 64% of the acetylated compound and suggested the possibility of the development of similar calculi in human beings receiving treatment.

Biochemical experiments

Each mouse in 6 groups of 10 mice was given 10 mg. doses of the selected drug on 3 successive days, whilst 3 mice were killed on the 2nd and 3rd days and survivors on the 7th day. There were no fatalities due to the drugs. A group of 10 normal mice was kept under the same conditions and killed at the same time for purposes of comparison. The animals were killed by severance of the

[1] Working under a full-time grant from the Medical Research Council.

spinal cord in order that the blood composition should not be changed. Heart blood was then taken under paraffin, and estimations of glucose, O_2 and CO_2 capacities and free and total sulphanilamide (or sulphapyridine) were made. The results are summarized in Table 1. Glucose was estimated by the Folin-Wu colorimetric method.

Table 1. *Blood results in relation to drug concentration (acetylated drug calculated as free for ease of comparison)*

Drug	Day	Drug conc. mg./100 ml. Free	Drug conc. mg./100 ml. Total	Glucose mg./100 ml.	O_2 capacity vol.	CO_2 capacity vol.
Nil	1	—	0·0	85	16·7	57
	3	—	0·0	82	16·4	55
	7	—	0·0	79	16·5	58
Sulphanilamide	2	18·0	32·5	70	16·5	50
	3	31·0	56·0	63	16·8	45
	7	2·0	3·6	83	16·1	52
Sulphanilamide + acetate	2	15·0	34·5	81	16·3	58
	3	29·0	67·0	80	16·3	63
	7	3·0	6·9	84	16·6	55
Acetylsulphanilamide	2	1·3	6·5	83	16·7	51
	3	2·7	10·8	79	16·3	45
	7	0·2	3·2	78	16·1	48
Sulphapyridine	2	11·0	16·5	75	16·9	52
	3	18·0	27·0	67	16·6	47
	7	8·7	13·0	80	16·7	54
Sulphapyridine + acetate	2	13·3	28·3	82	16·5	56
	3	21·0	46·3	80	16·4	57
	7	10·6	23·3	85	16·6	54
Acetylsulphapyridine	2	1·0	5·1	84	16·7	53
	3	2·1	9·8	79	16·8	50
	7	0·5	3·7	80	16·5	51

It will be seen that the O_2 capacity has not been affected by the drugs but that glucose and CO_2 have varied slightly, both falling in the absence of acetate and rising as the drug is excreted.

Sulphapyridine affects the blood picture in the same way as sulphanilamide does; acetate administration nullifies the depressant effect of both drugs.

The acetyl derivatives of the two drugs behave in a similar manner to each other and, whilst there is a slight fall in blood sugar, there is a marked fall of CO_2 capacity. The simultaneous administration of an acetate does not affect the toxicity of the acetylsulphanilamide [James, 1939] and so no attempt was made to examine the blood picture after the administration of acetates and acetylated drugs.

These results confirm the supposition first made that sulphanilamide kills by sudden withdrawal of acetate precursors whilst the acetyl compound exerts a specific effect. That this is not due to its lower solubility leading to blockage of the kidney tubules, such as occurs with acetylsulphapyridine, is shown by the histological results.

Histological results

The livers, spleens and kidneys of mice which had died as a result of acute toxic experiments with sulphanilamide, sulphanilamide and acetate and acetylsulphanilamide were examined histologically. No structural damage to the tissues was observed. This was confirmed by Dr R. M. Fry, Pathologist of these Laboratories.

Discussion

The use of acetate when administering drugs of the sulphanilamide class offers advantages in modifying the acute toxicity of sulphanilamide [James, 1939] and these experiments show that it acts by preventing the metabolism of acetate precursors. Other substances, such as lactate and bicarbonate, have been suggested to prevent the fall in CO_2 capacity. Acetate has this effect as well as providing acetyl radical directly; in addition an alkaline urine is produced in which sulphanilamide is known to be most bactericidal.

It is known that the acetyl compound is more toxic in mice, but as yet there is no evidence of its toxicity in man, although the acetyl derivative takes a considerable time to eliminate and some individuals may acetylate an extra large quantity.

The fall in glucose can be explained by glucose being an acetate precursor; this hypothesis is strengthened by the fact that when acetate is given this fall is prevented.

Summary

Both sulphanilamide and sulphapyridine have been found to be toxic due to withdrawal of acetate precursors and lowering of the CO_2 capacity of the blood. Acetate given at the same time rectifies this defect.

Acetylsulphanilamide evidently exerts a specific toxic effect although it must also have some effect on the respiratory centre on account of the fall of CO_2.

My thanks are due to Dr L. Colebrook for performing the animal experiments and to Dr R. M. Fry for confirming the histological findings.

REFERENCES

Antopol & Robinson (1939). *Proc. Soc. exp. Biol., N.Y.*, **40**, 428.
Beckmann (1938). *C. Clin. Invest.* **17**, 537.
Fuller (1937). *Lancet*, **1**, 194.
Gross, Cooper & Lewis (1939). *Proc. Soc. exp. Biol., N.Y.*, **40**, 448.
Hartmann, Perley & Barnett (1938). *J. clin. Invest.* **17**, 465.
Helmholz & Osterberg (1937). *Proc. Mayo Clin.* **12**, 661.
James (1939). *Biochem. J.* **33**, 1688.
Lucas & Mitchell (1939). *Canad. med. Ass. J.* **40**, 27.
Marshall, Cutting & Emerson (1937). *Science*, **85**, 202.
—— —— —— (1938). *J. Amer. med. Ass.* **110**, 252.
Ocklerblad & Carlson (1939). *J. Urol.* **41**, 801.
Southworth (1937). *Proc. Soc. exp. Biol., N.Y.*, **36**, 58.
Strauss & Southworth (1938). *Johns Hopk. Hosp. Bull.* **63**, 41.

77. THE OXIDATION OF SULPHANILAMIDE AND SULPHAPYRIDINE BY HYDROGEN PEROXIDE

By G. V. JAMES[1]

From the Bernhard Baron Research Laboratories, Queen Charlotte's Hospital, Hammersmith, W. 6

(*Received 23 January 1940*)

A PURPLE pigment was isolated from the urine of patients treated with sulphanilamide and from the urine of two patients who had received sulphapyridine. Since the pigments were probably oxidation products of the drugs, the following chemical oxidation experiments were undertaken to determine if the *in vitro* changes resembled those occurring *in vivo*.

Colebrook & Purdie [1937] diagnosed cyanosis in 53 cases and detected methaemoglobin or sulphaemoglobin in 45 of them, leaving 8 patients in whom cyanosis might have been due to pigment formation from the drug.

Marshall & Walzl [1937] found methaemoglobin in only 2 of 7 cyanosed patients receiving sulphanilamide. These authors obtained a black oxidation product by the action of calcium hypochlorite on sulphanilamide and attempted unsuccessfully to isolate a pigment from dark-coloured bloods taken from cyanosed patients in whom methaemoglobin was absent.

Ottenberg & Fox [1938] were led by the helio-sensitivity of patients treated with sulphanilamide to try the effect of ultraviolet light on dilute sulphanilamide solutions; brief exposure to the light produced a strong violet colour accompanied by a 30–60 % diminution of the sulphanilamide content of the solution. The violet colour was absorbed by red blood cells and the authors considered that the colour of the red cells of patients undergoing therapy might have a similar origin.

Harris & Michel [1939], in a survey of sulphanilamide cyanosis, observed in several samples a diffuse darkening of the blood with maximum absorption in the red at 670 mμ. Methaemoglobin and sulphaemoglobin were absent from these samples and the pigment faded in several hours even at 0°. The colour of the plasma was normal so the pigment was neither haematin nor methaemalbumin.

In the first experiments, hydrogen peroxide and calcium hypochlorite of equivalent oxygen availability were added to sulphanilamide solutions which were prepared in physiological saline, buffered at *p*H 7·4. Some of the solutions were left exposed to light at laboratory temperature (about 17°), others were kept in the dark at the same temperature and others were incubated in the dark at 38°. The solutions containing hydrogen peroxide in the dark at laboratory and at body temperatures did not lose sulphanilamide; the others did as shown in Table 1.

A red insoluble pigment was deposited from the solution containing peroxide. The solution treated with hypochlorite went through the same change but finally a violet or black pigment was deposited. The use of hypochlorite was discontinued in favour of peroxide plus small amounts of iron catalyst (Fenton's

[1] Working under a full-time grant from the Medical Research Council.

Table 1. *Change of concentration of sulphanilamide produced by oxidation with 6·5 ml. O_2 per 100 ml.*

Time in days	H_2O_2 17° light		$Ca(OCl)_2$ 17° light		$Ca(OCl)_2$ 17° dark		$Ca(OCl)_2$ 38° dark	
0	20·0	30·0	20·0	30·0	20·0	30·0	20·0	30·0
1	6·5	17·8	2·6	3·3	4·7	7·4	8·4	12·8
2	5·3	7·2	2·0	2·1	3·2	5·2	6·3	4·6
4	3·3	4·5	1·9	2·0	2·4	3·2	5·2	2·3
5	2·1	3·3	2·0	2·1	2·0	3·3	3·8	1·8
6	2·2	3·3	1·9	1·9	1·8	2·5	2·2	1·9

reagent), which more closely resembles the oxidizing system of the body. The effects of this combination on both sulphanilamide and sulphapyridine at room temperature with exposure to light are summarized in Table 2.

Table 2. *The catalytic effect of iron*

Time hr.	Sulphanilamide, mg. per 100 ml. H_2O_2		Sulphanilamide, mg. per 100 ml. H_2O_2 + 5 mg. $FeSO_4$		Sulphapyridine, mg. per 100 ml. H_2O_2		Sulphapyridine, mg. per 100 ml. H_2O_2 + 5 mg. $FeSO_4$	
0	20·0	40·0	20·0	40·0	20·0	40·0	20·0	40·0
1	19·4	39·8	18·8	38·4	20·1	39·5	19·0	38·6
2	19·2	39·2	15·8	37·8	19·6	38·7	19·0	38·8
3	18·9	38·5	13·2	33·2	19·3	37·5	19·2	34·1
4	18·2	37·8	12·7	26·7	18·8	35·3	17·0	28·2
24	15·2	32·8	1·8	23·0	16·5	34·1	15·0	25·0
48	8·5	28·6	1·9	7·0	13·3	22·5	8·1	20·0
96	6·6	23·0	1·6	6·8	9·3	18·3	7·2	17·0
240	2·2	6·4	1·5	6·2	6·7	10·8	6·5	12·1

The use of iron speeds up the reaction but with the same concentrations the same end point is reached without iron. The more rapid disappearance of sulphanilamide noted in Table 1 may be due to the larger amount of daylight falling on the solution, as the experiments recorded in Table 2 were done later in the year than the preliminary investigations of Table 1.

The results in Table 1 were obtained by simple diazotization as it was not then realized that this also estimated hydroxylamine compounds and it is possible that the action of light might lead to the further decomposition of hydroxylaminobenzene sulphonamide to *p*-aminophenol and other compounds; it is unlikely that peroxide in the dark is entirely without action on amines.

The experiments of Table 1 were repeated on sulphanilamide, hydroxylamine being estimated by adding 1 ml. acetic anhydride to 5 ml. of the solution, shaking well and allowing to stand for 5 min., then adding $N/2$ HCl and diazotizing in the usual manner. The acetic anhydride converts the amino group, if present, into the inactive acetamino group and the nitrous acid changes the hydroxylamine group to the amino group which is then estimated. To estimate the sulphanilamide, the hydroxylamine value is deducted from the value obtained for the first estimation which includes both amine and hydroxylamine.

p-Aminophenol may be estimated by the indophenol reaction the pigment being evaluated by placing the coloured solution in one cell of a Spekker photoelectric absorptiometer and taking the calibrated drum reading as a measure of the colour present. A combination of Ilford screens Micro 1 and 2 was used as being complementary to the pigment colour.

Table 3. *Effect of light on the production of hydroxylamine compound*

	17°				38°	
	Light		Dark		Dark	
Time days	Sulphanil-amide	Hydroxyl-amine	Sulphanil-amide	Hydroxyl-amine	Sulphanil-amide	Hydroxyl-amine
0	20·0	0·0	20·0	0·0	20·0	0·0
1	18·1	0·5	19·6	0·4	18·7	0·8
2	16·0	0·6	18·9	0·7	16·2	3·6
3	2·8	4·0	16·0	4·0	15·6	4·0
4	1·8	3·7	15·8	3·6	14·4	5·1
7	2·0	4·0	14·0	3·6	12·7	6·2

Table 4. *Estimation of all products of oxidation at 17° in daylight*

All results are expressed as mg. per 100 ml.

Time hr.	Sulphanil-amide	Hydroxyl-amine	*p*-Amino-phenol	Pigment drum reading	Sulpha-pyridine	Hydroxyl-amine	*p*-Amino-phenol	Pigment drum reading
0	40·0	0·0	0·0	0·000	40·0	0·0	0·0	0·000
1	39·7	0·0	0·0	—	39·6	0·0	0·0	—
2	39·2	0·0	0·0	—	39·0	0·0	0·0	—
3	38·6	0·8	0·0	—	38·0	0·0	0·0	—
4	38·0	1·2	0·0	0·000	37·2	0·0	0·0	—
24	32·0	3·4	0·0	0·077	32·0	2·4	0·0	0·105
48	25·0	8·5	1·0	—	18·2	5·3	2·6	0·176
72	21·1	10·2	2·6	0·228	14·4	6·1	3·2	0·245
96	15·0	12·1	5·8	0·302	12·1	5·9	5·3	0·389
120	3·2	11·9	4·1	0·348	4·6	7·8	5·1	0·541

Table 3 gives the results obtained by estimating sulphanilamide and hydroxylamine after oxidation in the light and in the dark at 17° and 38°. Table 4 shows the results of oxidation of the drugs with peroxide in terms of hydroxylamine compound, *p*-aminophenol and pigment in each case.

It is seen from Table 4 that the sum of the amounts of the drugs and of their oxidation products does not equal the original amounts of the drugs; the discrepancy becomes increasingly larger with time and corresponds to an increased colour in the solution.

The pigment from these solutions, and others in which it was prepared by the action of calcium hypochlorite, was separated by evaporating the solutions to dryness, extracting with alcohol, again evaporating, extracting the residue with ether and evaporating the ethereal solution.

The pigments were examined according to Rota's scheme for the examination of dyestuffs; the sulphanilamide pigments fell into the indophenol class, the same one as that of the pigment already isolated from the urine of sulphanilamide-treated patients. The sulphapyridine pigments could not thus be classified although they were reduced in the same manner as the pigments from sulphanilamide.

The separated pigments were soluble in blood fat and were absorbed on to washed red blood cells, imparting to them a chocolate colour such as may be seen in the blood of some patients who exhibited cyanosis.

The oxygen capacities of corpuscles with and without absorbed pigment were measured. It will be seen from Table 5 that the presence of the pigment does not affect the oxygen capacity. This is in accordance with the findings of Marshall & Walzl [1937].

Table 5. *Effect of pigment on O_2 capacity of blood*

	Original blood ml.	Blood + pigment ml.
Sulphanilamide pigment	11·8	11·6
Sulphapyridine pigment	14·82	14·97

Examination of alcoholic solutions of pigments with a hand spectroscope showed absorption in the red and blue regions. Red cells on which pigment had been absorbed showed no extra bands in the red, but in order to separate the two oxyhaemoglobin bands greater dilution of the blood was necessary owing to the non-specific absorption.

Discussion

The pigments obtained by oxidation of sulphanilamide and sulphapyridine can be absorbed on to red blood corpuscles and produce an effect resembling that of blood from some cyanosed patients. The chemical nature of the pigments is still obscure but they arise as a result of oxidation of the drugs, sulphapyridine producing more pigment and less intermediate products than sulphanilamide.

The fluctuations of hydroxylamine and *p*-aminophenol shown in Table 4 are probably due to further oxidation of these compounds to pigment which appears at the same time as, or a little later than, they do, and which in the case of sulphanilamide is an indophenol pigment.

Light is necessary for the *in vitro* oxidation of the hydroxylamine compound but *in vivo* this is achieved by other agencies.

Summary

Pigments have been obtained by oxidation of sulphanilamide and sulphapyridine which are absorbed by the blood fat of the cell envelope and this alters the colour of the blood without affecting the oxygen capacity or the spectrum. These pigments are the probable cause of cyanosis when both methaemoglobin and sulphaemoglobin are absent.

REFERENCES

Colebrook & Purdie (1937). *Lancet*, **2**, 1237.
Harris & Michel (1939). *J. clin. Invest.* **18**, 507.
Marshall & Walzl (1937). *Johns Hopk. Hosp. Bull.* **51**, 140.
Ottenberg & Fox (1938). *Proc. soc. exp. Biol., N.Y.*, **38**, 479.

78. THE ISOLATION OF SOME OXIDATION PRODUCTS OF SULPHANILAMIDE FROM URINE

By G. V. JAMES[1]

From the Bernhard Baron Research Laboratories, Queen Charlotte's Hospital, Hammersmith, W. 6

(*Received 23 January 1940*)

OXIDATION is a primary detoxicating mechanism of the organism; the first stage is often the formation of a hydroxyl derivative, which is then conjugated and excreted as an ethereal sulphate or glycuronate. Such oxidation products are formed after the administration of aromatic amines such as aniline, and it has become of interest to determine whether similar products are formed from sulphanilamide, and what part they play in the therapeutic activity of the compound.

Jaffé & Hilbert [1888] isolated *p*-aminophenol and *o*-hydroxycarbanil and its acetyl derivative from urine after acetanilide administration. Herrick & Irons [1906] found *p*-aminophenol in the urine after acetanilide poisoning, and further found an increase in the ethereal sulphates.

Young & Wilson [1926] confirmed this and also showed an increase in the blood phenols. They attributed the observed cyanosis to the production of a dyestuff which coloured the blood corpuscles.

Ellinger [1920] isolated acetylphenylhydroxylamine from the blood of cats, following single acetanilide injections.

Hinsberg & Treupel [1894] concluded that primary and secondary aminophenols could produce methaemoglobin, while their acetyl derivatives were less active and the ethers inactive.

Heubner [1913; 1923], investigating the action of *p*-aminophenols, suggested that unstable quinonimine was an intermediate in methaemoglobin formation.

Alsted [1937], Gley [1937] and Mayer [1937] have all suggested by analogy the possibility of the formation of *p*-hydroxylaminobenzenesulphonamide and *p*-aminophenol. Mayer, indeed, suggests that the whole anti-bacterial activity is due to the formation of oxidation products. This paper makes no attempt to investigate that phase of the problem, but confines itself to the description of compounds obtained at various stages of the chemical oxidation of sulphanilamide.

Sulphanilamide is an aromatic amine, but stabilized to some extent by the sulphonamide grouping in the para position. Sulphanilic acid, however, may be oxidized to *p*-quinone, the end-product of the oxidation of aniline. Like aniline, sulphanilamide causes methaemoglobinaemia, but with less frequency. It is possible that similar intermediates are formed in the oxidation of the two substances in the body, and the attempt was therefore made to isolate them from urine.

[1] Working under a full-time grant from the Medical Research Council.

Examination of urine for evidence of conjugation

The ratio of inorganic to ethereal sulphates was determined by the method of Rosenheim & Drummond [1914] before, during and after sulphanilamide therapy, when this was possible. Many patients, however, were not available before medication began, or for sufficiently long after it had ceased.

The results in Table 1 show that the sulphate ratio during medication falls below the normal value of 10 : 1 [Hawk & Bergeim, 1938], giving evidence of the production of hydroxy substances during administration of sulphanilamide.

Table 1. *Ratio of inorganic to ethereal sulphates*

The figures in parentheses are days over which the average was taken.

Patient	Before treatment	During treatment	After treatment
1	—	3·6 (5)	—
2	—	2·8 (2)	10·0 (4)
3	6·2 (3)	2·9 (7)	8·7 (2)
4	6·1 (1)	2·8 (7)	7·0 (1)
5	—	3·4 (10)	7·0 (4)
Average	6·2 (4)	3·1 (31)	8·2 (11)

Glycuronic acid was estimated in some of the urines by the method of Quick [1924]. It does not occur in normal urines and its presence is additional evidence of the formation of hydroxyl derivatives from sulphanilamide.

It was noticed that the urine of two patients with urinary infections contained less than the urine of a patient with a streptococcal infection. This may be due to decomposition of the glycuronic acid by organisms of the coliform group observed by Kay [1926] and by Quick & Kahn [1929]. The results of the estimations are shown in Table 2. As before, the figures in parentheses are the days over which the average was taken.

Table 2. *Excretion of glycuronic acid; mg. per 100 ml.*

Patient	Infection	Before treatment	During treatment	After treatment
2	*Bact. coli*	—	4·2 (2)	0·8 (4)
4	*Bact. coli*	0·0 (1)	2·8 (7)	3·1 (1)
5	*Strep. haem.*	—	10·5 (10)	5·9 (4)
Average		0·0 (1)	7·0 (19)	3·3 (9)

The presence of glycuronic acid and the altered sulphate ratio lead to the conclusion that conjugation is occurring and that hydrolysis will be necessary in order to liberate any oxidation products.

Production of methaemoglobin by urine

Neither normal urine nor that from patients receiving sulphanilamide, when incubated with defibrinated human blood for 18 hr. gave spectroscopic evidence of the presence of methaemoglobin.

When the urines were hydrolysed by boiling with conc. HCl for 15 min. and neutralized before incubating with the blood, the normal urine was still inactive, but the sulphanilamide urine caused very strong methaemoglobin bands to appear. The hydrolysis produces free hydroxylamine derivatives and also liberates *p*-aminophenol from its conjugation products.

Isolation of oxidation products

The increased ethereal sulphate and glycuronic acid in sulphanilamide urine and the failure to produce methaemoglobin except after hydrolysis all showed the advisability of hydrolysis prior to fractionation of the urine.

The urine was evaporated at 40° under reduced pressure, and the residues were then brought to *p*H 1·0 by the addition of HCl; a further addition of 5 ml. conc. HCl per 100 ml. residue was made to compensate for the loss caused by the decomposition of the urea present. The resulting liquid was then autoclaved at 15 lb. pressure per square inch (120°) for 2 hr. in a pressure bottle.

The *p*H of the final product varied from 3·0 to 5·0 and was adjusted to 5·0. Extraction with ether was then carried out several times. The aqueous residue still produced methaemoglobin when neutralized and incubated with blood, hence further ether extractions were made at *p*H 7·0 and 9·0, until finally the aqueous residue no longer produced methaemoglobin.

At this stage it was observed that the aqueous residue after extraction at *p*H 7 no longer produced methaemoglobin if the urine were from uncyanosed patients whilst if it were from cyanosed ones, then extraction at *p*H 9·0 was necessary for complete removal.

As a control specimens of urine from 8 normal puerperal patients who were not receiving sulphanilamide or its derivatives were processed, but none of the extracts produced methaemoglobin.

The ethereal extracts were separately evaporated and the residues so obtained were purified by recrystallization from water after treatment with activated charcoal. The partially purified products were again treated with activated charcoal and boiled for ½ hr., filtered, recrystallized and the crystals dried at 110°. Pale yellow or white solids were obtained which were submitted to further examination.

Examination of urine extracts

*Substance A, extracted at p*H *5·0.*

The purified residue was strongly acid in reaction and contained C, H, N, O and S. It gave a diazo reaction after reduction. The equivalent weight by titration with NaOH was 97 and it contained 7·2 % N and 17·2 % S; M.P. >300° with decomposition; it gave a violet colour with ferric chloride and benzoyl chloride (i.e. contained a hydroxylamine group).

These properties correspond to a substance of the formula $NHOH.C_6H_4.SO_3H$, mol. wt. 189, equiv. wt. 94·5 since the NHOH group is titratable with NaOH, it contains 7·4 % N and 17·0 % S.

*Substance B, extracted at p*H *7·0.*

The substance was faintly acid; it gave no diazo reaction either directly or after reduction; equiv. wt. by titration, 228·6; it contained 12·6 % N and 14·7 % S; M.P. >300° with decomposition; it gave no hydroxylamine reaction but after boiling with dilute HCl and reducing it was possible to couple the compound. These properties correspond with those of the substance $COCH_3.NH.C_6H_4.SO_2NH_2$ which is monobasic and has a mol. wt. of 230 and contains 11·6 % N and 13·2 % S.

This compound was prepared by acetylation of *p*-hydroxylaminobenzenesulphonamide; the M.P. of the latter was >300° with decomposition and its properties were similar to those of the compound isolated. This substance is analogous with the acetylphenylhydroxylamine isolated by Ellinger [1920].

Substance C and pigment, extracted at p*H 9·0.*

(*a*) This substance was taken out of ethereal solution by dilute HCl, decolorized with charcoal and evaporated to crystallizing point when long needles separated which on analysis gave the following results: 9·0 % N, and 23·0 % Cl; M.P. 306° with decomposition. It gave a violet colour with ferric chloride and a blue indophenol reaction. The chlorine was ionized in solution which was neutral and the substance on treatment with chloroform and NaOH gave a carbylamine reaction for primary amines. The monoacetyl compound melted at 167°. These properties correspond to *p*-aminophenol, the hydrochloride of which melts at 304° (decomposition) and contains 9·65 % N and 24·4 % Cl and the monoacetyl derivative of which melts at 168°.

(*b*) The residual ethereal solution was coloured purple although some pigment had been removed by the acid extraction. The solution was evaporated, leaving a tarry residue which could not be crystallized, although some purification was effected by adsorption on to charcoal and elution by absolute alcohol.

The pigment appeared to belong to the indophenol group since it was decolorized by stannous chloride to give a leuco base, and was regenerated by oxidation with ferric chloride [Rota, 1898]. The ethereal solution did not give up its colour to 5 % acetic acid and the colour changed to red after gentle warming with dilute HCl. Dr C. Rimington kindly examined a sample of this pigment and stated that it was not a porphyrin.

Extraction of p*-aminophenol from unhydrolysed urine*

Phenylhydroxylamine is converted into *p*-aminophenol by heating with HCl and an unsuccessful attempt was made to convert *p*-hydroxylaminobenzenesulphonamide into *p*-aminophenol under the conditions of hydrolysis. It was nevertheless desirable to isolate *p*-aminophenol from unhydrolysed urine.

p-Aminophenol was administered to mice and the urine collected to discover the form in which it is excreted and the best method of isolating it. The following method was adopted.

The urine was evaporated to a syrup which was extracted with 80 % alcohol, the extract filtered and the filtrate evaporated to a syrup and allowed to stand in a refrigerator. Crystals separated; the liquid was poured off and the crystals were rapidly washed with alcohol and dissolved in water. They gave positive diazo and indophenol reactions. After hydrolysis with dilute acid there were strongly positive sulphate and indophenol reactions but only a weak diazo reaction. The crystals were probably $NH_2.C_6H_4.OSO_3H$ showing that the substance was excreted principally as the acid sulphate.

This substance was also obtained from concentrated urines from patients receiving sulphanilamide who had shown cyanosis.

It was found that both the hydroxylamine compound and sulphanilamide formed compounds with xanthydrol by the method of Wood [1937], the hydroxylamine derivative melting at 260° with decomposition, whilst that of sulphanilamide melted at 208° (Wood found 209° for this compound).

When an alcoholic solution of xanthydrol was added to urine made acid with acetic acid and allowed to stand, crystals were obtained which were fractionated from 50 % acetic acid. The least soluble fraction on further crystallization and drying had a melting point of 258° while the more soluble fraction on similar purification melted at 208°. This showed the presence of free hydroxylamine compound and also of free sulphanilamide in the urine.

Xanthydrol did not form a condensation product with *p*-aminophenol.

Effect of cyanosis

The urine of a cyanosed patient was collected in two periods, firstly, during and immediately after cyanosis, secondly up to the cessation of medication. The urines were fractionated and the crude fractions weighed.

Table 3. *Yields of derivatives from the urine of a cyanosed patient*

			Substance A		Substance B		Substance C	
Period	Days	Vol. urine/day	mg./day	mg./100 ml.	mg./day	mg./100 ml.	mg./day	mg./100 ml.
1	10	663	120	18	45	7	29	4
2	2	112	200	18	82	7	12	1

Table 3 shows that while much less of substances A and B was passed per day during cyanosis, much more of substance C was excreted. This connexion between *p*-aminophenol and cyanosis has been repeatedly noticed.

Bactericidal properties of the substances

The bactericidal activity of the compounds was examined by Mr W. R. Maxted who found that substances A and B were bactericidal to group A haemolytic streptococci in whole blood, whilst substance C and *p*-aminophenol were not but produced methaemoglobin. In serum both C and *p*-aminophenol were bactericidal and produced a purple indophenol pigment soluble in serum but extracted by absolute alcohol. The failure of these substances to be bactericidal in whole blood is due to their interaction with the haemoglobin.

Estimation of the substances

It was found possible to estimate the *p*-aminophenol by the indophenol reaction and the hydroxylamine compound by the method of Pucher & Day [1926]. Making use of these estimations it was found that the sulphanilamide excreted in various forms approximated to 100 % of the amount given. Marshall *et al.* [1937, 1] found 70–87 % recovery of the drug in patients with normal renal function but only 12–57 % in patients with impaired renal function. The renal condition of the following cases was not investigated but it will be seen that the total recovery is often much greater than that indicated by the amount of sulphanilamide.

Table 4. *Recovery of sulphanilamide and its oxidation products, expressed as % of dose given*

Patient	Sulphanilamide %	Hydroxylamine as sulphanilamide %	*p*-Aminophenol as sulphanilamide %	Total recovery %
1	55·7	24·3	2·3	82·3
2	30·0	16·0	1·0	47·0
3	33·8	24·8	0·3	58·9
4	82·5	4·5	0·0	87·0
5	74·9	10·5	0·1	85·5
6	42·0	11·2	0·3	53·5

Discussion

The isolation of the oxidation products of sulphanilamide presents some difficulties, for they are very reactive substances and are excreted in the conjugated form with these reactive groups protected.

p-Acetylhydroxylaminobenzenesulphonamide (substance B) definitely occurs in the urine, and substance A, *p*-hydroxylaminobenzenesulphonic acid, is probably derived from it during the acid hydrolysis of the urine.

This view was confirmed, for substance A was obtained when either *p*-acetylhydroxylamino- or *p*-hydroxylamino-benzenesulphonic acid was hydrolysed with acid in the autoclave.

There is no certain evidence whether the hydroxylamine compound is excreted entirely in the acetylated form or whether some occurs free. At the time when this work was done, it was not known that simple diazotization also estimates the free hydroxylamine substance. Repeated failure had resulted from attempts to obtain increased sulphanilamide values from urine after reduction and this had been ascribed to the absence of the hydroxylamine compound.

The free compound cannot be present in large quantities, since unhydrolysed urine does not produce methaemoglobin. The isolation of the xanthydrol compound from untreated urine, on the other hand, suggests that it does occur in the free state, although even in this experiment the use of 50 % acetic acid may have produced some of it by hydrolysis.

It may not be as stable to oxidation as substance A (*p*-hydroxylaminobenzenesulphonic acid), and may have been oxidized further to the azoxy compound and beyond. Other oxidation products of the azoxy and quinone types are probably present in the urine, but these, if present, were destroyed during hydrolysis or removed by the extensive charcoal treatments. Polymerization and condensation of these products to give tarry substances probably occurs.

This investigation is only of a preliminary character and a more thorough fractionation might yield other substances.

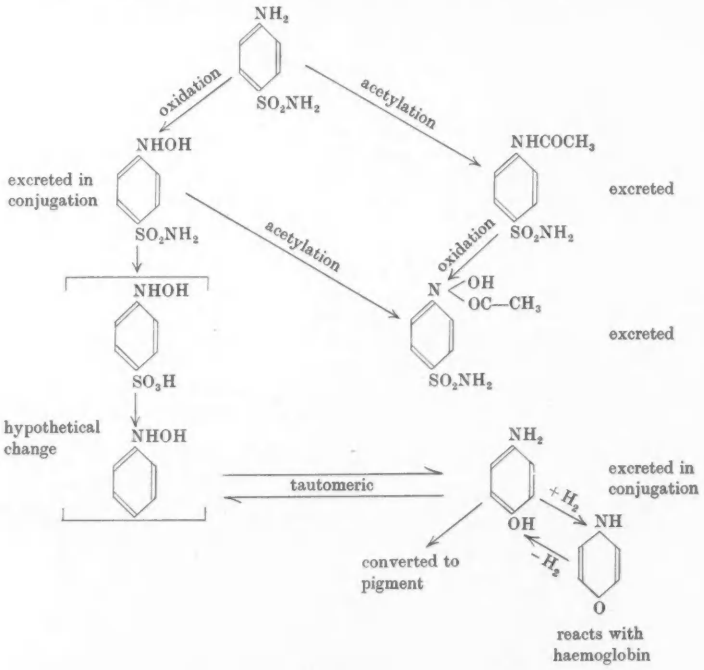
The *p*-aminophenol is probably present in the urine in the conjugated form, for there was evidence of its ethereal sulphate in untreated urine. The failure of the urine to produce methaemoglobin or to give an indophenol reaction before hydrolysis shows that free *p*-aminophenol is not present. There is a possibility that some is derived from the other oxidation products during the acid hydrolysis of the urine. Phenylhydroxylamine yields *p*-aminophenol by heating with acid but it was not detected in control experiments after acid hydrolysis in the autoclave of hydroxylaminobenzenesulphonamide or its acetyl derivative. The conversion is probably hindered by the presence of the sulphonamide grouping in the para position.

Some of the *p*-aminophenol is excreted with the amino group free and the phenol group conjugated, for it gives the diazo reaction, but hydrolysis leads to a negative diazo test owing to oxidation of the free *p*-aminophenol by the nitrous acid.

The pigment of the indophenol group is interesting in view of the theory of Young & Wilson [1926] that a dyestuff was the cause of cyanosis in aniline poisoning and of the isolation of a pigment from the blood cells by Lundsten *et al.* [1938] after acetanilide poisoning. Marshall *et al.* [1937, 2], however, were unable to isolate a pigment from the blood of patients treated with sulphanilamide. The production of pigments by oxidation of sulphanilamide has been reported by many authors and illustrates the complexity of the substances that may be formed by the condensation of the primary oxidation products.

The stages of sulphanilamide oxidation and excretion may be represented as follows [cf. Rimington & Hemmings, 1939], although future knowledge may clarify some reactions which at the moment are obscure.

The oxidation products may be the cause of some of the toxic effects noticed by clinicians. Both the hydroxylamine compound and *p*-aminophenol in the unconjugated form can produce methaemoglobin. The hydroxylamine derivatives may be the cause of the drug rash, since phenylhydroxylamine causes eczema in susceptible persons [Gattermann & Wieland, 1937].



Summary

p-*N*-Acetylhydroxylaminobenzenesulphonamide, *p*-hydroxylaminobenzenesulphonic acid and *p*-aminophenol have been isolated from the urine of patients treated with sulphanilamide. They appear to be excreted in conjugation with sulphates and glycuronates. The *p*-aminophenol undergoes further change to give rise to a pigment which is also excreted.

It is suggested that these compounds may be the cause of some of the toxic effects noted during therapy with sulphanilamide.

The author wishes to express his thanks to Dr L. Colebrook for the animal experiments, to Mr W. R. Maxted for performing the bactericidal tests, to Dr C. Rimington for examining a sample of pigment submitted to him and to Dr A. T. Fuller for many helpful suggestions.

REFERENCES

Alsted (1937). Ugeskr. Laeg. No. 50, 1347.
Ellinger (1920). *Hoppe-Seyl. Z.* **121**, 86.
Gattermann & Wieland (1937). Laboratory Methods of Organic Chemistry, p. 175. Macmillan.
Gley (1937). *Bull. Acad. Méd. Paris*, **118**, 377.
Hawk & Bergeim (1938). Practical Physiological Chemistry, p. 628. Churchill.
Herrick & Irons (1906). *J. Amer. med. Ass.* **46**, 351.
Heubner (1913). *Arch. exp. Path. Pharmak.* **72**, 241.
—— & Meier (1923). *Arch. exp. Path. Pharmak.* **100**, 137.
Hinsberg & Treupel (1894). *Arch. exp. Path. Pharmak.* **33**, 216, 250.
Jaffé & Hilbert (1888). *Hoppe-Seyl. Z.* **12**, 295.
Kay (1926). *Biochem. J.* **20**, 321.
Lundsten, Meulengracht & Rischel (1938). *Acta med. scand.* **96**, 462.
Marshall, Emerson & Cutting (1937, 1). *J. Amer. med. Ass.* **108**, 953.
—— & Walzl (1937, 2). *Johns Hopk. Hosp. Bull.* **61**, 140.
Mayer (1937). *Biol. méd. Supplément*, **27**, 74.
Pucher & Day (1926). *J. Amer. chem. Soc.* **48**, 672.
Quick (1924). *J. biol. Chem.* **61**, 667.
—— & Kahn (1929). *J. Bact.* **18**, 133.
Rimington & Hemmings (1939). *Biochem. J.* **33**, 960.
Rosenheim & Drummond (1914). *Biochem. J.* **8**, 143.
Rota (1898). *Chem. Z.* **22**, 437.
Wood (1937). *Nature, Lond.*, **136**, 837.
Young & Wilson (1926). *J. Pharmacol.* **27**, 133.

79. THE ABSORPTION, CONVERSION AND THERAPEUTIC ACTION OF BENZYL-SULPHANILAMIDE

By G. V. JAMES[1] and A. T. FULLER

From the Bernhard Baron Research Laboratories, Queen Charlotte's Hospital, Hammersmith, W. 6

(*Received 23 January 1940*)

Colebrook *et al.* [1936] showed that sulphanilamide had a direct bacteriostatic action on the streptococcus, and that this constituted the essential action of the drug both *in vivo* and *in vitro*. This view was at first denied by other workers but is now generally accepted. To support this view and thus provide a rational explanation of the therapeutic action of the many drugs of the sulphanilamide type it is necessary to show that each of them either has a bacteriostatic action, or is changed in the body to a substance having such action.

Fuller [1937] attempted to do this for prontosil, which of itself has no bacteriostatic action, by a quantitative study of the sulphanilamide produced from it. The present paper is a quantitative study of the behaviour of benzylsulphanilamide (Proseptasine of May and Baker or M and B 125, Septazine of the Rhone-Poulenc Co.) which is another substance possessing therapeutic activity but no direct bacteriostatic action. We have shown that sulphanilamide is produced from it in the body, and that the same therapeutic effects are produced by administering benzylsulphanilamide or the quantity of sulphanilamide which is produced from it by the body. The question of the mode of absorption and conversion is also discussed.

Benzylsulphanilamide was first synthesized by Goissedet *et al.* [1936] and was described as less toxic than sulphanilamide by Halpern & Mayer [1937], who considered it to be as effective as prontosil or sulphanilamide in experimental streptococcal infections. Hoare [1939] did not find it to be so effective although some protection was afforded. On account of its insolubility its mode of action remained obscure, although Ewins [1937] informed us that he had been able to isolate unchanged benzylsulphanilamide from the urine of patients receiving the drug and there was also some evidence for alteration of the compound to give free sulphanilamide.

Molitor & Robinson [1939] thought that the sulphanilamide present in the blood of animals fed with benzylsulphanilamide might explain the therapeutic effect, and that by use of this drug a more constant blood sulphanilamide level was maintained. Lockwood & Robinson [1939] found that 75 mg. of benzylsulphanilamide were therapeutically equal to 12 mg. of sulphanilamide when the two were given in single daily doses, whilst when given every 4 hr. the equivalence was 18 mg. benzyl derivative to 2 mg. sulphanilamide. According to these workers a blood level of between 2 and 3 mg. per 100 ml. sulphanilamide was maintained in mice by giving 18 mg. of benzyl derivative every 4 hr.

[1] Working under a full-time grant from the Medical Research Council.

LINDA HALL LIBRARY
KANSAS CITY, MO.

Chemical characters of benzylsulphanilamide and methods employed for its estimation

Benzylsulphanilamide is a white, odourless powder, very slightly soluble in water, the solubility being increased by the addition of acid or alkali. The solubility in water at 37° varies with the fineness of the powder as shown in Table 1.

Table 1

	Solubility mg./100 ml.
Retained by 100 mesh	0·333
Retained by 200 mesh passing 100 mesh	0·325
Retained by 300 mesh passing 200 mesh	0·360
Retained by 350 mesh passing 300 mesh	0·390
Passing 350 mesh	0·426

The compound is very stable and is hydrolysed to free sulphanilamide with extreme difficulty; heating with 70% H_2SO_4 hydrolyses it to ammonia. It can be reduced to free sulphanilamide, this reduction being probably responsible for its conversion in the body. It may be oxidized to benzoic acid, but the yield is not quantitative; nitrous acid gives an insoluble yellow compound which has the characteristics of a nitroso compound but so far these last characteristics have proved of no value for its determination.

It was estimated in urine by the following method. The urine was saturated with sodium sulphate, adjusted to *p*H 7·0 and extracted with light petroleum for 8 hr. The petroleum was distilled off and the residual benzyl compound hydrolysed by heating in a water bath for 1 hr. with 70% H_2SO_4 and the resulting ammonia estimated by nesslerization.

Adopting this method, the following results were obtained in a series of unknowns, the benzylsulphanilamide being estimated with an accuracy of 5% in the presence of free and acetyl-sulphanilamide.

Table 2

Benzylsulphanilamide, mg./100 ml.		
Present	Found	% error
6·0	6·3	+ 5
5·9	6·2	+ 5
1·9	1·9	0
2·2	2·1	− 4·5
7·5	7·2	− 4·0
1·5	2·0	+33·5

Administration of benzylsulphanilamide to man

The drug was administered to human beings by mouth in a single dose using 0·5 g. tablets of coarse-particle size. The urine was only examined for free and acetyl-sulphanilamide.

Excretion commenced at the end of the 12th hr. and continued to the end of the 2nd day. As the dose was increased the percentage conversion dropped so that it is possible that very large doses would not greatly increase the amount of sulphanilamide produced. The results are shown in Table 3.

Mayer [1937] and Gournay & Le Balch [1937] have reported that absorption varies with particle size. We have confirmed this, using single 1 g. doses of powder passing 300 mesh on 3 subjects. With this fine powder it was found that excretion of sulphanilamide commenced after the 2nd hr., attained its maximum

Table 3

Dose, g.	0·5	1·0	2·0
Total sulph. produced, mg.	34·9	54·4	68·0
Benzylsulph. equivalent, mg.	54·4	81·6	102·0
% conversion	10·5	8·2	5·1

on the 2nd day and then decreased, persisting from 3 to 5 days in all, depending on the subject. Table 4 shows that the percentage conversion of the coarse powder or tablets was about half that of the fine powder.

Table 4. *Percentage benzylsulphanilamide converted by 3 subjects*

No. of subject	Coarse powder retained by 100 mesh gauze	Fine powder passing 300 mesh gauze
1	8·9	16·2
2	—	15·7
3	—	21·5
	Average	17·8

Absorption and conversion of benzylsulphanilamide

An attempt was next made to determine in what part of the alimentary canal conversion occurred. A stomach tube was passed and the fasting juice removed by a syringe. A dose of 1 g. fine benzylsulphanilamide mixed with some marmite (to stimulate the flow of juice) in warm water was taken; portions of the juice were withdrawn after 5 min. and after 15 min. Free sulphanilamide was absent from the juices and saliva even after incubation of these juices for 24 hr. at 37°. The conversion does not therefore take place in the stomach. When keratin-coated capsules containing 1 g. of fine mesh benzylsulphanilamide were given, examination of the urine showed that 11 % of the drug had been converted. This confirms the fact that alteration does not occur in the stomach.

Giving magnesium sulphate 4 hr. before and again 4 hr. after 1 g. of benzylsulphanilamide diminishes the excretion of the free sulphanilamide in the urine. This effect was probably due to diminished absorption of water from the small intestine and to more rapid passage of the intestinal contents.

In another experiment an attempt was made to line the intestine with liquid paraffin by giving a dose of 30 ml. It was found that the conversion was thereby not greatly diminished (14·5 %), possibly because the drug is more soluble in oils than in water and the liquid paraffin may be absorbed [Channon & Collinson, 1929]. The evidence on the whole suggests conversion in the small intestine.

Oral administration of benzylsulphanilamide to mice

10 mg. of the fine drug suspended in 0·5 ml. gum saline were administered by stomach tube to each of 10 mice whose urine and faeces were collected.

Table 5

	Urine Sulphanilamide, mg.			Faeces Benzylsulphanilamide mg.
	Free	Acetyl	Benzyl	
1st day	0·415	0·135	0·015	20·6
2nd day	0·580	0·108	0·011	46·0
3rd day	—	—	—	21·4
Total sulph.	0·995	0·243	—	—
Benzylsulph. equivalent	1·520	0·310	0·026	87·0
Total benzylsulph.	—	—	1·860	87·0

Free sulphanilamide could be detected in the urine after 4 hr. and was present until the end of the 2nd day. Most of the drug was excreted unchanged in the faeces by the 3rd day. Detailed results are given in Table 5.

Since 100 mg. had been given there was a recovery of 89% of which 87% was in the faeces and 1·9% in the urine as sulphanilamide.

Comparison of conversions in normal and infected mice

Two groups each of 15 mice were dosed with 20 mg. benzylsulphanilamide suspended in 0·5 ml. gum saline per mouse on each of 4 successive days. One group was infected with Robb strain of group A haemolytic streptococcus 2 hr. before the first dosing with the drug. Urine and faeces were collected; in both groups of mice the main excretion of benzylsulphanilamide was in the faeces. After 3 days the infected mice ceased to pass urine and so no comparison of conversions could be made with the normal mice. Mice were killed off from each group for the examination of their blood and organs. The free blood sulphanilamide reached a maximum in 4 days and then diminished.

In another experiment a balance sheet showing the distribution and fate of the drug in normal mice was prepared. 20 mg. benzylsulphanilamide were administered to each of 5 mice on 4 successive days; urine and faeces were collected and after 6 days the group was killed for the estimation of benzylsulphanilamide in the organs etc. Each day a mouse from another similarly

Table 6

Average life of control, untreated, infected mice = 1·5 days
Average life of benzylsulphanilamide-treated, infected mice = 6·7 days

	Uninfected mice	Infected mice
Urine, mg. of converted benzylsulph.	0·78 or 1·1%	0·207 or 0·3%
Urine, mg. of unconverted benzylsulph.	0·012 or 0·0%	0·066 or 0·01%
Faeces, mg. unconverted benzylsulph.	39·7 or 58·0%	31·2 or 51·0%
Total dose benzylsulph. given, mg.	68·0	61·5

The difference in dosage is due to death of some of the infected mice, and the smaller urine excretion was due to urine retention by some of the infected animals prior to death.

Balance sheet of benzylsulphanilamide
Benzylsulphanilamide administered: 80 mg. fine or 300 mesh

	Converted mg.	Unconverted mg.	Total mg.	%
Urine	2	0·02	2·02	2·5
Faeces	—	74·0	74·0	92·9
Intestines	—	1·12	1·12	1·4
Spleen	—	0·02	0·02	0·03
Liver	—	0·06	0·06	0·09
Rest of body	—	0·42	0·42	0·53
Totals	2	75·64	77·64	97·55

Blood results: with fine powder. Free sulphanilamide, mg. per 100 ml.

	Uninfected mice			Infected mice
Days	1st exp.	2nd exp.	Average	
2nd	0·9	1·2	1·0	0·9
3rd	1·4	2·2	1·8	1·2
4th	1·8	2·8	2·3	2·4
5th	—	1·4	1·4	—
6th	—	0·9	0·9	0·5
9th	0·4	—	0·4	—
11th	0·1	—	0·1	—

treated group was killed in order to confirm the blood findings of the previous experiment. The results are detailed in Table 6, the average length of life of the mice being calculated by the method of Whitby [1937]. The results in this and subsequent tables are calculated to a basis of one mouse.

Subcutaneous administration

30 mg. of benzylsulphanilamide in 0·7 ml. of sterile gum saline were given on 2 successive days to each mouse of 2 groups, one group infected with Robb strain of streptococcus. Excreta were collected from both groups and mice from both groups were killed for blood examination.

By a separate experiment planned on similar lines to that recorded in Table 6, a balance sheet was drawn up indicating the fate of the compound. A comparison was also made of the result of using coarse or 100 mesh and fine or 300 mesh benzylsulphanilamide on uninfected mice. The results are included in Table 7. A portion of the injected drug was still present in the subcutaneous tissues after 3 weeks.

Table 7

Average life of control, untreated, infected mice = 1·2 days

Average life of benzylsulphanilamide-treated, infected mice = 11·8 days

	Uninfected mice	Infected mice
Urine, mg. of converted benzylsulph.	0·95 or 1·4%	1·85 or 3·3%
Urine, mg. of unconverted benzylsulph.	0·024 or 0·03%	0·045 or 0·08%
Faeces, mg. unconverted benzylsulph.	1·26 or 1·8%	1·3 or 2·3%
Total dose given, mg.	69·0	57·0

Balance sheet of benzylsulphanilamide

Dose given to each group, 70 mg.

	Fine or 300 mesh				Coarse or 100 mesh			
	Converted mg.	Unconverted mg.	Total mg.	%	Converted mg.	Unconverted mg.	Total mg.	%
Urine	2·8	0·04	2·84	4·0	0·52	0·02	0·54	0·7
Faeces	—	2·56	2·56	3·7	—	2·56	2·56	3·7
Tissue deposit	—	62·7	62·7	89·0	—	61·5	61·5	88·0
Intestines	—	0·7	0·7	1·1	—	0·74	0·74	1·1
Spleen	—	0·08	0·08	0·1	—	0·1	0·1	0·02
Liver	—	0·26	0·26	0·4	—	0·16	0·16	0·2
Rest of body	—	0·62	0·62	0·9	—	0·76	0·76	1·1
Totals	2·8	66·96	69·76	99·2	0·52	65·84	66·36	94·82

Blood results: free sulphanilamide, mg. per 100 ml.

	Uninfected mice				Infected mice
	Fine powder			Coarse powder	Fine powder
Days	1st exp.	2nd exp.	Average		
2nd	0·8	1·4	1·1	0·6	1·4
3rd	1·2	1·1	1·1	0·7	1·1
4th	2·3	1·7	2·0	1·3	1·7
5th	1·2	1·2	1·2	1·1	1·2
6th	—	0·9	0·9	0·7	—
7th	1·0	—	1·0	—	1·2
10th	0·4	—	0·4	—	0·3

The greater concentration of free sulphanilamide in the blood with the fine powder shown in Table 7 is another illustration of the greater conversion obtainable with the drug in this form.

Intraperitoneal administration

In this case only a balance experiment was attempted and no infected animals were used. The experiment was carried out in duplicate, 35 mg. of 300 mesh benzylsulphanilamide were given to each mouse on 2 consecutive days. The following results were obtained (Table 8).

Table 8. *Balance sheet for benzylsulphanilamide*

70 mg. benzylsulphanilamide given in each case

	I				II			
	Converted mg.	Unconverted mg.	Total mg.	%	Converted mg.	Unconverted mg.	Total mg.	%
Urine	2·46	0·09	2·55	3·0	4·5	0·04	4·54	6·5
Faeces	—	0·14	0·14	0·2	—	0·18	0·18	0·5
Tissue deposit	—	63·62	63·62	91·0	—	52·4	52·4	75·0
Intestine	—	0·3	0·3	0·4	—	0·04	0·04	0·1
Spleen	—	0·01	0·01	—	—	0·12	0·12	0·3
Liver	—	0·04	0·04	0·1	—	0·04	0·04	0·1
Rest of body	—	2·22	2·22	3·1	—	5·8	5·8	8·3
Total	2·46	66·42	68·88	97·8	4·5	58·62	63·12	90·8

Blood results days	Free sulphanilamide mg. per 100 ml.
2nd	1·2
3rd	1·8
4th	2·3
5th	2·0

Since the experiments in which the various methods of administration were used lasted for different periods of time, a calculation of the amounts converted during the first 8 days has been made and is given in Table 9; all the mice were uninfected and received 300 mesh benzylsulphanilamide. As before, the results are calculated for one mouse.

Table 9. *Comparison of routes of administration*

	Oral	Subcutaneous	Intraperitoneal
Dose, mg.	68·0	69·0	70·0
Excretion, mg.	1·07	0·96	1·57
% conversion	1·57	1·39	2·25

Comparison is difficult, but if conversion had occurred only in the intestines, a much greater conversion would have been expected in the case of the orally treated animals.

Conversion of benzylsulphanilamide in mice

Data from the balance sheets are compared in Table 10 where a similarity between the results of the different methods of administration is seen. The amount mobilized is the amount given less the amount found in the depot (the faeces, in the case of the oral method) whilst the amount recovered of the amount mobilized is the sum of all the benzylsulphanilamide found in the body and excreta but excluding that found in the depot.

The amounts found in the intestines are included in the amounts found in the body, since, when removed from the mice for analysis, the intestines were washed free from their contents which were added to and included with the faeces for analysis; the intestines probably contained the drug in the mucosa.

Table 10. *Conversion of benzylsulphanilamide in mice*

Method of dosage	Oral	Subcutaneous		Intra-peritoneal
	Fine	Fine	Coarse	Fine
Amount mobilized, mg.	6·0	7·3	8·5	12·0
Amount recovered of above, mg.	3·64	4·26	4·86	7·0
% recovered of the amount mobilized	60·7	58·3	57·1	58·3
Amount converted, mg.	2·0	2·8	0·5	3·5
% converted of the amount mobilized	33·0	38·3	6·0	29·1
Amount in body, mg.	1·64	4·22	4·32	4·5
Ratio: converted/recovered	1·82	1·5	0·95	2·0

The conversion in various organs was studied by taking organs from 2 groups of mice under sterile conditions, mixing the minced organs with silver sand and extracting them with Ringer-Locke glucose solution. These extracts were mixed with 20 mg. benzylsulphanilamide, incubated at 37° and examined at the end of 18 and 24 hr. for free and acetyl-sulphanilamide. The results are summarized in Table 11.

Table 11. *Distribution of sulphanilamide in the tissues, mg./100 ml.*

Organ	18 hr.			36 hr.		
	Free	Acetyl	Ratio acetyl/free	Free	Acetyl	Ratio acetyl/free
Group A:						
Spleen	0·2	0·08	0·4	0·23	0·09	0·4
Liver	0·2	0·06	0·3	0·22	0·07	0·3
Intestines	0·9	0·09	0·1	0·86	0·00	0·0
Group B:						
Spleen	0·25	0·05	0·2	0·3	0·09	0·3
Liver	0·3	0·03	0·1	0·25	0·2	0·8
Intestines	0·8	0·03	0·04	0·9	0·18	0·2

It will be seen that all the organs examined have produced free sulphanilamide from the benzyl derivative, but the intestines have produced the most, probably owing to the heavy bacterial growth normally present. As would be expected the liver has produced the most acetyl derivative (on the average).

From Table 10 it will be seen that the percentage recovered of the amount mobilized is about the same in all cases and the percentage converted of the amount mobilized is again very similar (except in the case of the coarse powder). This indicates that there is a similarity in the methods of conversion in each case.

A comparison of the results obtained respectively by the administration of fine and coarse powder subcutaneously shows that about the same amount was present in the body in each case; hence, about the same amount will enter the intestines, but the fact that there is about 6 times as much converted when the fine powder is used suggests that reduction probably occurs in the tissues as a whole and not only in the intestines. This is explained by the work of Hulett [1901] who showed that fine powdering reduces the work necessary for solution. Small particles show a greater solubility, heat of solution and also lower melting point.

The therapeutic effect of benzylsulphanilamide

Bactericidal experiments *in vitro* with benzylsulphanilamide have shown that it has no perceptible activity against haemolytic streptococci, whilst sulphanilamide is active. The rational explanation of the activity of benzyl-

sulphanilamide is that it is due to the liberated sulphanilamide. The work of Lockwood & Robinson [1939] and of Molitor & Robinson [1939] bore this out and suggested that free sulphanilamide was formed when the benzyl derivative was given to animals, but no quantitative measure of the sulphanilamide formed had been made.

To prove that sulphanilamide is the curative agent, it is necessary to show that the benzyl derivative and sulphanilamide itself are equally curative under conditions producing the same excretion and blood concentration of sulphanilamide; this is what we have done.

Infected mice receiving 80 mg. of benzylsulphanilamide in all excrete on the average 1 mg. of sulphanilamide per day, much less than the dose of 5 mg. per day which is the usual therapeutic dose of sulphanilamide. The daily dose of benzylsulphanilamide has however the advantage of providing a steady supply of free sulphanilamide for 48 hr., whereas much of the sulphanilamide is excreted in the first few hours. We spread the sulphanilamide dose by giving it orally every 8 hr. which gave a fairly steady excretion of sulphanilamide throughout the day. The repeated handling of the sick animals hindered their cure and with better means of ensuring a steady supply of free sulphanilamide, the protection from the 2 mg. per day of sulphanilamide would have been greater. The technique resolved itself into administering 2 mg. sulphanilamide in 0·5 ml. gum saline per day for 5 days, the 2 mg. being divided into 3 equal quantities administered at 8 hr. intervals to infected mice. The benzylsulphanilamide was administered in doses of 20 mg. suspended in 0·5 ml. gum saline on each of 4 days.

Table 12 summarizes the results obtained.

Table 12. *Curative effects on mice*

		Benzylsulphanilamide			Sulphanilamide		
Strain of mouse	No. of mice	Average life, days	14-day survivors	mg. sulph. excreted	Average life, days	14-day survivors	mg. sulph. excreted
L 193	10	5·6	3	38·1	11·1	6	35·0
Richards	8	14·0	8	38·6	11·5	3	24·5
Richards	8	11·0	4	32·6	10·8	1	28·1
	Averages	10·2	5	36·4	11·1	3·3	29·2

From these results it will be readily recognized that the therapeutic activity of the benzyl derivative is due to the liberated sulphanilamide and can be simulated by giving repeated small doses of sulphanilamide which produce and maintain the same blood concentration in both infected and uninfected mice. The correspondence would probably have been closer with smaller quantities given at shorter intervals as suggested by the results given in Table 13.

Table 13. *Excretion of sulphanilamide, mg.*

Time hr.	Benzylsulphanilamide		Sulphanilamide (small doses)	
	Infected	Uninfected	Infected	Uninfected
24	1·6	1·9	1·4	1·8
48	1·9	1·6	2·1	1·5
72	1·8	1·7	1·9	1·7

Discussion

Benzylsulphanilamide could be converted into free sulphanilamide by hydrolysis or by reduction. The benzyl group is easily split off by reduction and in the body the conversion almost certainly takes place in this way. This

reduction is probably brought about by the action of the intestinal flora and also occurs in the tissues. The facts that more extensive conversion occurs after intraperitoneal injection than after oral dosage and that the ratio of free sulphanilamide produced to benzyl derivative mobilized in the body is constant for all 3 methods of administration, suggest that the intestine cannot play a dominating part in the conversion.

The sulphanilamide derived from proseptasine is more economically employed than sulphanilamide given in one dose per day since sulphanilamide is a soluble drug which is rapidly excreted.

Benzylsulphanilamide gives rise to only low blood concentrations of sulphanilamide, insufficient to counteract severe infections, but capable of curing mild ones. Increasing the dose does not correspondingly increase the amount absorbed by the body. The more finely divided the benzylsulphanilamide the greater the quantity of sulphanilamide produced. For example, a fine or 300 mesh powder shows 5–6 times the conversion that a coarse powder does. It is probable that colloidal benzylsulphanilamide would give a still greater conversion.

Benzylsulphanilamide is, as reported by other workers, less toxic than sulphanilamide because so little is absorbed, and that part which is absorbed is almost entirely changed to free sulphanilamide which is used very economically by the body.

SUMMARY

Benzylsulphanilamide is converted in the body to sulphanilamide which is maintained at a low but steady concentration in the blood; the therapeutic activity of the drug is due to the sulphanilamide produced.

The authors wish to thank Dr Colebrook for performing the animal experiments and bactericidal tests and for many useful suggestions, and Messrs May and Baker, Ltd. for supplying the benzylsulphanilamide (proseptasine) used.

REFERENCES

Channon & Collinson (1929). *Biochem. J.* **23**, 676.
Colebrook, Buttle & O'Meara (1936). *Lancet*, **2**, 1323.
Ewins (1937). Personal communication.
Fuller (1937). *Lancet*, **1**, 194.
Goissedet, Despois, Gaillot & Mayer (1936). *C.R. Soc. Biol., Paris*, **120**, 756.
Gournay & Le Balch (1937). *Biol. méd. Supplément*, **27**, 6.
Halpern & Mayer (1937). *Pr. méd.* **45**, 747.
Hoare (1939). *Lancet*, **1**, 76.
Hulett (1901). *Z. physikal. Chem.* **37**, 385.
Lockwood & Robinson (1939). Personal communication.
Mayer (1937). *Biol. méd. supplément*, **27**, 74.
Molitor & Robinson (1939). *J. Pharmacol.* **65**, 405.
Whitby (1937). *Lancet*, **1**, 1517.

80. THE COLI-TRYPTOPHAN-INDOLE REACTION

3. ESSENTIAL STRUCTURAL CONDITIONS FOR THE ENZYMIC DEGRADATION OF TRYPTOPHAN TO INDOLE

By JOHN WILLIAM BAKER and FRANK CHARLES HAPPOLD

From the Department of Organic Chemistry and the Biochemical Laboratories, Department of Physiology, The University, Leeds

(*Received 18 March 1940*)

The reaction whereby *l*-tryptophan is broken down to indole by *E. coli* has been studied independently by Woods [1935, 1, 2] and by Happold & Hoyle [1935]. Woods worked with washed viable cells of the organism, whereas the latter investigators used suspensions killed in various ways but which, nevertheless, retained the property of catalysing this reaction. By coincidence both investigations, although following different courses, included examination of a similar selection of those indole derivatives which, on the basis of various mechanisms suggested in the literature, should be intermediate stages in the breakdown. Both sets of data (obtained by different techniques) uniformly revealed failure to produce indole from such derivatives and thus effectively disproved the validity of current mechanisms which, after all, had been formulated solely on analogy to other degradations.

Woods [1935, 2] also studied the kinetics of the reaction and showed that the process requires 5 atoms of oxygen, that the conversion of tryptophan into indole goes to completion, and that the rate of indole production is equal to the rate at which the tryptophan disappears. The reaction could be represented

$$C_{11}H_{12}O_2N_2 + 5O \rightarrow C_8H_7N + NH_3 + H_2O + 3CO_2$$

and thus involves an over-all oxidation, deamination and decarboxylation. Whether these processes are effected simultaneously by different enzymes or by a single master enzyme which catalyses simultaneously a number of reactions associated with the fission and oxidative breakdown of the alanine side-chain is not known.

The present communication describes an investigation in which the side-chain of tryptophan was modified systematically in an attempt to determine the structural features which are essential for the degradation of the molecule to indole by tryptophanase. The following derivatives have been tested with active enzyme systems: (CO_2H group modified) *l*-tryptophan methyl ester hydrochloride, *l*-α-amino-β-3-indolylpropionamide; (NH_2 group modified) the phenylurethane, *p*-nitrobenzoyl-, and *N*-methylene-derivatives of *l*-tryptophan, methyl *l*-α-methylamino-β-3-indolylpropionate hydriodide, *r*-α-methylamino-β-3-indolylpropionic acid, methyl-α-dimethylamino-β-3-indolylpropionate methiodide; (whole side-chain modified but still containing groups of the type NH_2 or NHR) β-3-indolylethylamine, 3-indolylacetamide, 3-indolealdehyde semicarbazone, 3-indolylglyoxylamide and, finally *r*-3-indolylglycine, the next lower homologue of tryptophan.

Experimental

Cultures of *E. coli* grown for 18 hr. on nutrient agar in two Roux bottles were each washed off with 40 ml. of normal saline and the cells separated by centrifuging. The deposit was resuspended in a similar amount of saline, the suspension recentrifuged and the deposits were finally suspended in 16 ml. of saline.

Tryptophan or the derivatives used were made up in *M*/20 phosphate buffer *p*H 7·5 and 1 mg. of the compound tested according to the following scheme.

A. 2 ml. washed cells, 20 ml. phosphate buffer *p*H 7·5, 10 ml. toluene and 1 mg. tryptophan.

B. 2 ml. washed cells, 20 ml. phosphate buffer *p*H 7·5, 10 ml. toluene and 1 mg. derivative.

C. 2 ml. washed cells, 20 ml. phosphate buffer *p*H 7·5, 10 ml. toluene.

D. 2 ml. boiled cells, 20 ml. phosphate buffer *p*H 7·5, 10 ml. toluene and 1 mg. tryptophan.

These four mixtures were aerated for 18 hr. at laboratory temperature (17–19°), extracted with 2 separate, 30 ml. portions of light petroleum (20–40°) and tested for indole by the method adopted by Happold & Hoyle [1934]. A similar series was incubated without toluene at 37° for periods up to 6 hr.

The toluene has the property of rendering the cells almost immediately nonviable but, since it also extracts the indole and the enzyme system is still appreciably active, the reaction tryptophan → indole goes almost to completion.

Preparation of derivatives

Modification of the α-amino group

Phenylurethane of l-*tryptophan* was prepared by the method of Abderhalden & Kempe [1907]. Crystallized from methyl alcohol it had m.p. 169° (decomp.) (*loc. cit.*, m.p. 166° (corr.)). (Found: C, 66·9; H, 5·1%. Calc. for $C_{18}H_{17}O_3N_3$: C, 66·8; H, 5·3%.)

p-*Nitrobenzoyl derivative of* l-*tryptophan*. 0·5 g. of *l*-tryptophan, 2·5 ml. of *N* NaOH and 0·46 g. of *p*-nitrobenzoyl chloride dissolved in a little ether were shaken together for 1·5 hr., two further additions, each of 2·5 ml. of *N* NaOH, being added at intervals. Yellow crystalline material (probably the required derivative) soon separated but dissolved again when the further quantity of alkali was added. The solution was extracted with ether (which removed only a small amount of a brown crystalline material, m.p. *c.* 213°) and the alkaline solution was acidified with a little conc. HCl which precipitated a brown oil. This was extracted with ether. The residue from the dried ethereal extract slowly solidified to a glass-like mass. Crystallization of this from ethyl acetate-ligroin (b.p. 40–60°) gave *p*-nitrobenzoic acid, m.p. 228–232° (raised to 237° by admixture with a genuine specimen). The red syrup obtained by concentration of the mother liquor was diluted with warm 96% alcohol and seeded with a small crystal which had been obtained in an attempt to crystallize the original mixture from this solvent. The whole mass crystallized: it was triturated with a little cold 96% alcohol and drained on porous pot. Crystallization from 96% alcohol afforded the *p-nitrobenzoyl* derivative, sinters 114°, m.p. 121° (decomp.) in clusters of slender prisms. The compound seems to contain one molecule of alcohol of crystallization which is not completely eliminated at 120° in a vacuum. (Found: C, 60·0; H, 5·4; N, 10·85%. $C_{18}H_{15}O_5N_3 + C_2H_5OH$ requires C, 60·1;

H, 5·3; N, 10·5%. Loss in weight when heated at 120°/vac. = 9·6%. Loss for 1 mol. EtOH = 11·4%. Found on the residue: C, 60·6; H, 4·3%. $C_{18}H_{15}O_5N_3$ requires C, 61·2; H, 4·7%.)

Methylene derivative of l-*tryptophan*. This was obtained by Homer's method [1913]. It was found to be simpler to dissolve the *l*-tryptophan directly in 40% formalin solution with the aid of gentle warming. When the solution is kept at 38° ready crystallization of the compound occurs. Contrary to Homer all samples obtained had M.P. 196° (Homer [1913] gives M.P. 235–240°) not raised by washing with boiling acetone. The analysis figures of the compound dried in a vacuum at room temperature, when the specimen remained colourless, or at 110–120° for several hours, when it became slightly yellow, do not agree well with the compositions of the dihydrate and monohydrate, respectively, assigned by Homer. (Found, for a vacuum-dried sample: C, 58·5; H, 6·3; N, 10·0%. Calc. for $C_{12}H_{12}O_2N_2, 2H_2O$: C, 57·1; H, 6·35; N, 11·1%. Found for a sample dried at 110–120°: C, 62·3; H, 6·1; N, 12·7%. Calc. for $C_{12}H_{12}O_2N_2, H_2O$: C, 61·5; H, 6·1; N, 12·0%.) Such discrepancies raise doubts whether the action of formaldehyde upon *l*-tryptophan is as simple as the structures assigned by Homer suggest.

Methyl l-α-*methylamino-β-3-indolylpropionate hydriodide*. A well dried specimen of *l*-tryptophan methyl ester hydrochloride (0·6 g.) was suspended in dry ether and dry NH_3 was passed into the solution at 0°. The precipitated NH_4Cl was filtered off and the free ester, obtained by evaporation of the solution, was well dried in a desiccator. It was then redissolved in dry ether and 0·24 g. of methyl iodide was added. The stoppered vessel was kept at room temperature for 24 hr. An oil separated on the sides of the vessel and slowly crystallized after standing for several days. The solution was decanted and the hydriodide was repeatedly washed *in situ* with dry ether. Crystallization of the solid residue from a very concentrated solution of dry acetone cooled in a refrigerator gave a small amount of the *hydriodide*, decomp. 192°. Microtitration with $AgNO_3$ gave I 34·8%. $C_{13}H_{16}O_2N_2$, HI requires I, 35·3%.

The specimen of *r*-α-methylamino-β-3-indolylpropionic acid was one synthesized by Miller & Robson [_938], and we wish to express our gratitude to Dr Robson for this generous gift.

Methyl-α-dimethylamino-β-3-indolylpropionate methiodide was obtained by methylation of *l*-tryptophan as described by van Romburgh & Barger [1913]. After crystallization from boiling water the specimen had M.P. 198° (decomp.). (Found: C, 46·0; H, 5·2%. Calc. for $C_{15}H_{21}O_2N_2I$: C, 46·4; H, 5·4%.)

Modification of the carboxyl group and of the side-chain as a whole

l-*Tryptophan methyl ester hydrochloride*, M.P. 214°, was prepared by the method of Abderhalden & Kempe [1907]. (Found: C, 57·0; H, 6·0%. Calc. for $C_{12}H_{14}O_2N_2$, HCl: C, 56·6; H, 5·7%.)

l-*Tryptophan amide*, M.P. 165–167° (capillary tube) was a specimen specially synthesized by Prof. C. P. Berg [cf. Baugess & Berg, 1934] to whom we wish to express our deep gratitude for his generous gift and help.

β-*Indolylethylamine hydrochloride* was purchased from Schuchardt.

β-*Indolylacetamide*. Preparation of the amide of β-indolylacetic acid through the acid chloride was found to be unsatisfactory since the action of either thionyl chloride or of phosphorus pentachloride in acetyl chloride on the acid gave amorphous, highly coloured products. It was obtained, however, by thermal decomposition of the ammonium salt. β-Indolylacetic acid (British Drug Houses) was purified by repeated crystallization from dry benzene and a

sample, M.P. 170°, was converted into its ammonium salt by evaporation of a solution of 1 g. of the acid in concentrated aqueous ammonia. The dry ammonium salt was mixed with a little ammonium carbonate and heated in a glycerol bath at 200–210° until gas evolution ceased (15 min.). The cooled residue was extracted with warm, dry benzene. The sparingly soluble amide, M.P. 135–140°, crystallized out when this solution was concentrated. Repeated crystallization from benzene containing a little alcohol gave the *amide*, M.P. 150–151°. (Found: C, 68·6; H, 5·9; N, 15·6%. $C_{10}H_{10}ON_2$ requires C, 69·0; H, 5·8; N, 16·1%.) Evaporation of the benzene mother liquors afforded the very soluble skatole, M.P. 90–91°, obtained by decarboxylation of the acetic acid.

Indole-β-aldehyde [Boyd & Robson, 1935] could not be made to give any aldehyde-ammonia compound, but forms a *semicarbazone*, M.P. 220° (decomp.). after crystallization from absolute alcohol. (Found: C, 59·0; H, 5·1. Calc. for $C_{10}H_{10}ON_4$: C, 59·4; H, 5·0%.)

The synthesis of 3-indolylglyoxylamide, M.P. 252°, and *r*-3-indolylglycine, M.P. 221° (decomp.) have been described by one of us elsewhere [Baker, 1940].

Results and discussion

None of the derivatives examined as substrates for tryptophanase gave rise to the formation of indole with the exception of tryptophan methyl ester hydrochloride and tryptophanamide. The former is certainly only an apparent exception, since the data in Table 1 prove conclusively that the indole produced arises from the breakdown of *l*-tryptophan which is first formed by the hydrolysis of the ester in the slightly alkaline medium employed.

Table 1. *Indole formation from* l-*tryptophan methyl ester hydrochloride at 37°*

Indole formation after	0·5	1·0	2 hr.
(*a*) From fresh ester hydrochloride	0·022	0·070	0·078 mg.
(*b*) From ester hydrochloride previously left for 18 hr. in the medium at *p*H 7·5	0·070	0·118	0·095 mg.
(*c*) From *l*-tryptophan	0·070	0·118	0·088 mg.

The results obtained with *l*-tryptophanamide are interesting: they are summarized in Table 2.

Table 2. *Indole formation from* l-*tryptophanamide at 37°*

A. Tryptophanamide just brought to boil in medium *p*H 7 and left for 2 hr. in medium at 37° before addition of the washed cells.

B. Tryptophanamide previously left for 2 hr. in medium *p*H 7 at 37°.

C. Tryptophanamide added immediately to cells.

D. *l*-Tryptophan control.

Indole formed after	0·75	1·5	3 hr.
A	0·003	0·09	0·10 mg.
B	0·005	—	0·110 mg.
C	Faint trace	0·042	0·089 mg.
D	0·04	0·15	0·15 mg.

From these results it can be concluded:

(*a*) That the production of indole from the amide occurs very much more slowly (C) than from tryptophan itself (D) especially in the early stages of the reaction.

(*b*) That pre-treatments (A and B) expected to effect some preliminary hydrolysis of the amide, increase the initial rate of indole production, which, however, is still much less than that from tryptophan itself (D).

(*c*) That the reaction which produces indole from the amide is autocatalytic. Thus after 3 hr. (C) has given 0·089 mg. of indole which approximates closely to the amount (0·09 mg.) obtained after $2+1{\cdot}5=3{\cdot}5$ hr. in (A).

It would thus appear probable that indole production again really occurs through the breakdown of tryptophan itself, but that part of the enzyme complex may be able to adapt itself to the enzymolysis of the amide to tryptophan, thus enabling the tryptophanase reaction to proceed. It has been reported that the coli bacillus possesses deamidases [Uttjino & Imazumi, 1938] so that such a preliminary enzymic deamidation would readily account for these results.

The side-chains which, when attached to the 3-position in the indole nucleus, have now been shown not to undergo degradation to indole with tryptophanase, are summarized in Table 3.

Table 3. *Modifications of the alanine side-chain in* l-*tryptophan which fail to undergo degradation to indole*

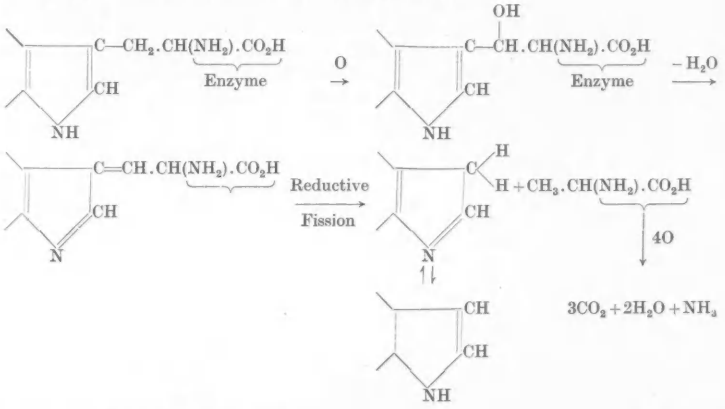
	Side-chain	Reference
I. α-NH_2 group modified	$.CH_2.CH(NHMe).CO_2H$	B.H.
	$.CH_2.CH(CO_2H).N^+Me_3)I^-$	,,
	$.CH_2.CH(CO_2H).N{:}CH_2$	,,
	$.CH_2.CH(NHR).CO_2H$	,,
	(R = .CO.NHPh and $.CO.C_6H_4.NO_2-p$)	
II. CO_2H group modified	$.CH_2.CH(NH_2).CO_2CH_3$	B.H.
III. Whole side chain modified	$.CH_2.CH_2.CO_2H$	W., H.H.
	$.CH{:}CH.CO_2H$	,,
	$.CH_2.CH(OH).CO_2H$	,,
	$.CH_2.CO.CO_2H$	,,
	$.CH_2.CO_2H$	,,
	$.CH_2.CO.NH_2$	B.H.
	$.CO_2H$	W., H.H.
	.CHO	,,
	$.CH{:}N.NH.CO.NH_2$	B.H.
	$.CH_2.CH_2NH_2$	B.H., Frieber
	$.CH(NH_2).CO_2H$ (*dl*-)	B.H.
	$.CO.CO.NH_2$	,,

B.H. = this communication.
W. = Woods [1935, 1, 2].
H.H. = Happold & Hoyle [1935].
Frieber [1922].

These data would seem to afford fairly conclusive evidence that the integrity of both the α-amino and carboxylic acid groups in the alanine side-chain is essential for the enzymic breakdown of tryptophan to indole.

The experimental exclusion of indole-3-carboxylic acid as the penultimate stage in this production of indole suggests that the latter may be produced by the elimination of the alanine side-chain as a whole. It is significant that the lower homologue of tryptophan, *r*-3-indolylglycine, does not give indole. This suggests that the breakdown mechanism may involve oxidation at the β-carbon atom of the alanine side-chain. The presence of a suitable oxygen substituent on this β-carbon renders possible prototropic change in the indole ring with consequent conversion into the indolylidene structure. If a parallel reductase system were present in the enzyme indole might then be formed by the direct reductive fission of this indolylidene structure. The essential integrity of the

α-amino and carboxylic acid groups might be needed to "fix" an amphoteric enzyme system to the tryptophan molecule, and in this connexion, it is perhaps not without significance that the optimum production of indole occurs in a medium of approximately *p*H 7·5. The following scheme is illustrative of the general type of breakdown tentatively suggested:



It is recognized that such reductive fission is a very unusual reaction and has no known analogy, nor must it be assumed that the fission occurs directly in one stage.

Amongst the derivatives examined, only in four (with the side-chains —CH:O, .CH:N.NH.CO.NH_2, —CO.CO.NH_2 and .CH:CH.CO_2H) is prototropic conversion into an indolylidene form structurally possible. None of these derivatives retains, in addition, the essential integrity of both the carboxyl and α-amino groups, and in no case is indole produced.

The next step in the experimental testing of the very tentative mechanism proposed is obviously to ascertain whether replacement of the mobile hydrogen in the indole ring by an alkyl group is, alone, sufficient to prevent the breakdown to indole; but since present circumstances make it probable that further attack on the problem might have to be postponed, the results already obtained have been placed on record.

Summary

The action of tryptophanase on a large number of new tryptophan derivatives in which the alanine side-chain has been systematically modified has been studied.

The results obtained suggest that the breakdown to indole requires, *inter alia*, the following structural features:

(*a*) a free carboxyl group;
(*b*) an unsubstituted α-amino group;
(*c*) a β-carbon atom capable of oxidative attack.

A tentative mechanism for the breakdown is suggested.

REFERENCES

Abderhalden & Kempe (1907). *Hoppe-Seyl. Z.* **52**, 207.
Baker (1940). *J. chem. Soc.* 458.
Baugess & Berg (1934). *J. biol. Chem.* **106**, 615
Boyd & Robson (1935). *Biochem. J.* **29**, 555.
Frieber (1922). *Centr. Bakt.* **87**, 254.
Happold & Hoyle (1934). *Biochem. J.* **28**, 1171.
—— —— (1935). *Biochem. J.* **29**, 1918.
Homer (1913). *Biochem. J.* **7**, 105.
Miller & Robson (1938). *J. chem. Soc.* p. 1910.
van Romburgh & Barger (1913). *J. chem. Soc.* **99**, 2096.
Uttjino & Imazumi (1938). *J. Biochem. Tokyo*, **27**, 257.
Woods (1935, 1). *Biochem. J.* **29**, 640.
—— (1935, 2). *Biochem. J.* **29**, 649.

81. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

65. (A) A SURVEY OF CHLORINE METABOLISM BY MOULDS

(B) CALDARIOMYCIN, $C_5H_8O_2Cl_2$, A METABOLIC PRODUCT OF *CALDARIOMYCES FUMAGO* WORONICHIN

BY (THE LATE) PERCIVAL WALTER CLUTTERBUCK, SUDHIR LAL MUKHOPADHYAY, ALBERT EDWARD OXFORD AND HAROLD RAISTRICK

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London

(*Received 20 March 1940*)

THREE mould metabolic products containing chlorine have recently been described: geodin, $C_{17}H_{12}O_7Cl_2$, and erdin, $C_{16}H_{10}O_7Cl_2$, from *Aspergillus terreus* Thom, strain No. 45 [Raistrick & Smith, 1936; Clutterbuck *et al.* 1937; Calam *et al.* 1939] and griseofulvin, $C_{17}H_{17}O_6Cl$, from *Penicillium griseo-fulvum* Dierckx [Oxford *et al.* 1939].

In view of these findings a quantitative survey has now been made of the chlorine metabolism of 139 species or strains of moulds grown on the well-known Czapek-Dox 5% glucose solution containing 0·5 g. KCl per litre as the sole source of chlorine. The organisms studied included 27 species of *Aspergillus*, 96 species of *Penicillium* and species in the following genera: *Absidia* (1); *Alternaria* (2); *Botrytis* (1); *Caldariomyces* (3); *Cephalosporium* (1); *Cladosporium* (1); *Clasterosporium* (1); *Helminthosporium* (1); *Mucor* (1); *Stemphylium* (1); *Stysanus* (1); *Syncephalastrum* (1) and *Trichoderma* (1).

The results obtained show that extensive conversion of inorganic chloride into organic chlorine-containing metabolic products is of rather rare occurrence. Of the 139 species or strains examined, 57 utilized less than 5% of the KCl originally present in the medium; 77 utilized from 5 to 25% of the KCl and certain species in this group might well repay further investigation since it includes *Penicillium griseo-fulvum*, the species giving griseofulvin; only 5 cultures comprising three different species metabolized more than 25% of the KCl. Two of these were different strains of *Aspergillus terreus* Thom including strain No. 45 which gives geodin and erdin, one was *Absidia spinosa* Lendner which is being investigated at present and the remaining two were different strains of *Caldariomyces fumago* Woronichin, an investigation of which is described in Part B of the present communication. An interesting feature of this species is the fact that it also metabolizes considerable amounts of bromine in a medium containing KBr in place of KCl. Although no bromine-containing metabolic products of *Caldariomyces fumago* have at present been obtained in a state of purity, crude mixtures of metabolic products containing considerable amounts of bromine in organic combination have been isolated.

Caldariomyces fumago, which was formerly known as *Fumago vagans* Persoon, is one of the common "sooty" greenhouse moulds. A considerable part of the chloride metabolized by this species appears in the metabolism solution as a hitherto undescribed substance which we propose to name *caldariomycin*.

Caldariomycin, $C_5H_8O_2Cl_2$, forms colourless needles, M.P. 121°, which are dextrorotatory in aqueous solution. The molecular constitution suggested for caldariomycin illustrates the following experimentally established facts.

(1) It contains no methoxyl or side-chain methyl groups.

(2) Its two chlorine atoms are particularly labile since they are almost quantitatively liberated as chloride when a cold solution of caldariomycin in *N*/10 NaOH is kept overnight.

(3) It contains no ketonic or aldehyde groups but, since it has two "active" hydrogen atoms (Zerewitinoff), the probable presence of two actual or potential hydroxyl groups is indicated though no satisfactory derivatives proving the presence of these groups have been obtained.

(4) It is oxidized by cold aqueous chromic acid with the formation of succinic acid thus proving the presence of the grouping $\rangle C—CH_2.CH_2—C\langle$.

(5) It is reduced by hydrogen and a palladium-charcoal catalyst to *cyclo*pentanone, C_5H_8O. Hence one may justly argue that caldariomycin is a derivative of *cyclo*pentane, C_5H_{10}, so that the five carbon atoms in its molecule are accounted for.

(6) Assuming, as indicated in (3), the presence of two hydroxyl groups then it is clear that these cannot be attached to adjacent carbon atoms as the grouping $\rangle C(OH).C(OH)\langle$ since caldariomycin is not attacked by the Malaprade [1928] reagent, i.e. HIO_4, in acid solution. This belief is strengthened by the fact that if the hydroxyl groups were attached to adjacent carbon atoms caldariomycin would be a derivative of *cyclo*pentane-1:2-diol, but both high- and low-melting forms of *cyclo*pentane-1:2-diol are known and have been prepared and shown to be oxidized by the Malaprade reagent, the low-melting form to the extent of 40% and the high-melting form almost quantitatively.

(7) It is very stable to heat and does not lose either H_2O or HCl at moderate temperatures. Above 180°, however, it decomposes to give, along with H_2O, HCl and black resinous products, a mixture of two isomeric ketones, each having the empirical formula C_5H_5OCl and each giving a mono-2:4-dinitrophenylhydrazone. One of these ketones is also formed when a solution of caldariomycin in 2*N* H_2SO_4 is boiled.

(8) It does not contain the grouping $—CH_2.CO—$ since it gives no ketonic reactions. This grouping is however formed on treatment with dilute caustic alkali since such a solution now gives a precipitate with Brady's reagent (2:4-dinitrophenylhydrazine in 2*N* HCl). Further, caldariomycin itself does not immediately give the Zimmermann reaction for an active CH_2 group as modified by Callow *et al.* [1938] though an alkaline solution of caldariomycin, after standing for some time, quickly gives an intense reaction. Finally, although caldariomycin reduces Fehling's solution in the cold this reduction is only apparent after a considerable lag period during which a reducing substance is presumably formed in the strongly alkaline solution.

The above experimental facts indicate clearly that caldariomycin is a dichlorodihydroxy*cyclo*pentane. No dichlorodihydroxy*cyclo*pentanes are described in the literature but of the many possible structural isomerides only three (I, II and III) will satisfy the following four essential conditions.

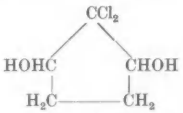
(*a*) The presence of at least one asymmetric carbon atom.

(*b*) The presence of the grouping $>C.CH_2.CH_2.C<$ to account for the succinic acid formed on oxidation.

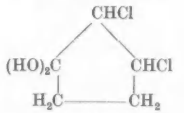
(*c*) The absence of the grouping $>C(OH).C(OH)<$.

(*d*) The absence of the grouping

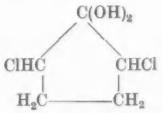
since a compound containing this grouping, even if stable in the solid state, would almost certainly give free HCl at once in aqueous solution.



I



II



III

II and III are essentially hydrated forms of two different dichloro*cyclo*pentanones but cannot be excluded on that account alone since a $>CO$ group so hydrated might not react with Brady's reagent (cf. chloral hydrate $CCl_3.CH(OH)_2$ which does not give the characteristic aldehyde reactions).

The main objection to II and III is that they provide no explanation for the comparatively great stability of caldariomycin towards heat. At least two solid hydrates of polychloro-aldehydes and -ketones are known: (*a*) chloral hydrate which boils at 97·5° to yield a vapour phase containing both chloral and water molecules; (*b*) *sym*tetrachloroacetone tetrahydrate, $CHCl_2.CO.CHCl_2, 4H_2O$, which loses all its water at 110–115° and distils in the anhydrous state without decomposition at 180° [Levy & Jedlicka, 1888]; the hydrate is also completely dehydrated on standing over conc. H_2SO_4 [Zincke & Kegel, 1889]. Since caldariomycin does not begin to lose water on heating until a temperature of 180° is reached and can even be distilled unchanged at 149–150° at 20 mm. it seems improbable that it is a hydrate of a dichloro*cyclo*pentanone. By a process of elimination I is therefore indicated as the most probable structure for caldariomycin, which is thus probably *2:2-dichloro*cyclo*pentane-1:3-diol.*

I offers a simple explanation for almost all the experimental facts available including the formation of the two isomeric ketones, C_5H_5OCl, referred to above under (7). It does not, however, give such a simple explanation for the formation of *cyclo*pentanone on catalytic reduction as do II and III, i.e. by mere replacement of both Cl atoms by H and subsequent loss of 1 mol. of water. Accepting I, a possible mechanism for this reaction is the following: replacement of one Cl atom by H followed by loss of water to give a chlorohydroxy*cyclo*pentene, the double bond in which is reduced to give a chlorohydroxy*cyclo*pentane which, by loss of HCl, gives the enolic form of *cyclo*pentanone.

It is worthy of note that in all four chlorine-containing metabolic products so far reported—geodin, erdin, griseofulvin and caldariomycin—chlorine atoms are always found adjacent to hydroxyl or methoxyl groups in a grouping of the form $>C(Cl)—C(OR)<$. This fact suggests that a reaction analogous to that by which ethylene chlorohydrin, $CH_2Cl.CH_2OH$, is formed by the action of dilute hypochlorous acid on ethylene, $CH_2=CH_2$, may possibly serve to account for the formation of chlorine-containing metabolic products by moulds.

Experimental

Part A. A survey of chlorine metabolism by different species of fungi

The medium used throughout this part of the investigation was the well-known Czapek-Dox solution having the following composition: glucose, 50 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4$, $7H_2O$, 0·5 g.; $FeSO_4$, $7H_2O$, 0·01 g.; distilled water, 1 litre. As the object of the work was to follow quantitatively the chloride remaining in solution at different stages of growth particular care was taken to ensure that each flask of medium contained the same amount of chloride. This was done as follows. All the constituents of the medium except the KCl, and in amount sufficient for 35 litres of medium, were dissolved in 32·5 litres of distilled water and 325 ml. amounts were measured into each of 100 one-litre conical flasks. 25 ml. of a 0·7 % aqueous solution of KCl were now accurately measured, by pipette, into each flask. All the flasks were plugged with cotton wool and sterilized. Batches of four flasks were sown with 2·5 ml. of a spore suspension, in 10 ml. of distilled water, of a Czapek-Dox or beer-wort agar culture of the mould under investigation. The flasks were incubated in the dark at 24°.

The glucose content (by polarimeter) and KCl content (by Volhard method) of duplicate unsown flasks from each batch of 100 flasks were estimated. At suitable intervals, so arranged that in the last of the four flasks examined for each species the glucose had been almost completely metabolized, the chloride remaining in solution was estimated as follows. The metabolism solution under investigation was filtered through a Büchner funnel and the mycelium was thoroughly washed with hot distilled water. The filtrate and cooled washings were made up to 500 ml. and duplicate estimations of the chloride present in aliquot portions were carried out by the method described by Raistrick & Smith [1936, p. 1316] which involves the removal of interfering substances with norite charcoal and estimation of chloride in the purified solution by the Volhard method. This technique has been proved to give accurate and reproducible results. Residual glucose (by polarimeter) was also estimated in all metabolism solutions.

The results obtained are summarized below. The species examined are arranged in groups according to the degree of chloride metabolized. Only the *maximum* amount of chloride metabolized by each species is recorded and is expressed as % of the chloride originally present. The figures in brackets following the name of the species examined denote (*a*) the incubation period in days in which chloride absorption reached its maximum, and (*b*) the residual glucose on this day.

Group I. *Metabolized 0–1 % of the total chloride originally present.* 7 species = 5 % of all species examined. *Aspergillus tamarii* Kita (85; 0·0 %); *Penicillium brunneo-violaceum* Biourge (19; 0·18 %); *P. Charlesii* G. Smith (36; 0·0 %); *P. expansum* Link em. Thom (42; 0·05 %); *P. puberulum* Bainier (38; 0·0 %); *P. schneggii* Boas (24; 3·94 %); *Alternaria* sp. (70; 2·28 %).

Group II. *Metabolized 1–2 % of the total chloride originally present.* 14 species = 10 % of all species examined. *Penicillium aurantio-candidum* Dierckx (68; 3·65 %); *P. aurantio-griseum* Dierckx var. *poznaniensis* Zaleski (54; 0·0 %); *P. aurantio-virens* Biourge (67; 2·10 %); *P. brunneo-rubrum* Dierckx (strain P. 9) (64; 0·81 %); *P. citreo-viride* Biourge (25; 0·34 %); *P. crateriforme* Abbott (33; 2·35 %); *P. cyaneo-fulvum* Biourge (strain P. 34) (22; 1·26 %); *P. Dierckxii* Biourge (42; 0·08 %); *P. jantho-citrinum* Biourge (23; 0·92 %); *P. olivino-viride*

Biourge (24; 0·29 %); *P. purpurogenum* Fleroff-Stoll (24; 0·29 %); *P. spiculisporum* Lehman (25; 1·58 %); *P. subcinereum* Westling (strain P. 20) (61; 0·0 %); *P. viridi-dorsum* Biourge (25; 1·31 %).

Group III. *Metabolized 2–3 % of the total chloride originally present.* 21 species = 15 % of all species examined. *Aspergillus repens* (Corda) Saccardo (57; 0·81 %); *A. terreus* Thom (strain Ac. 24) (60; 0·60 %); *Penicillium atramentosum* Thom (67; 0·11 %); *P. Biourgei* Dierckx (20; 1·76 %); *P. Brefeldianum* Dodge (41; 1·33 %); *P. chrysogenum* Thom (strain P. 18) (64; 0·08 %); *P. citreosulfuratum* Biourge (strain P. 59) (65; 0·08 %); *P. citrinum* Thom (strain P. 25) (25; 0·29 %); *P. decumbens* Thom (70; 0·0 %); *P. digitatum* Saccardo (42; 1·47 %); *P. fellutanum* Biourge (strain P. 67) (54; 0·0 %); *P. flavido-marginatum* Biourge (20; 0·08 %); *P. frequentans* Westling (strain P. 29) (39; 0·0 %); *P. griseo-fulvum* Dierckx (strain P. 38) (64; 1·1 %); *P. notatum* Westling (23; 0·65 %); *P. patrismei* Zaleski (22; 0·29 %); *P. roqueforti* Thom (strain P. 54) (25; 2·10 %); *P. subcinereum* Westling (strain P. 21) (65; 0·21 %); *P. terlikowskii* Zaleski (67; 0·18 %); *Cephalosporium* sp. (49; 3·15 %); *Helminthosporium monoceras* Drechsler (70; 4·97 %).

Group IV. *Metabolized 3–5 % of the total chloride originally present.* 15 species = 11 % of all species examined. *Aspergillus candidus* Link (84; 0·0 %); *A. flavus* Link (77; 0·0 %); *A. niger* van Tieghem (strain No. 33) (48; 0·0 %); *A. oryzae* (Ahlburg) Cohn (84; 0·0 %); *A. terreus* Thom (strain Ac. 100) (77; 0·0 %); *A. versicolor* (Vuillemin) Tiraboschi (strain No. 18) (46; 0·05 %); *Penicillium Adametzi* Zaleski (64; 0·08 %); *P. cinerascens* Biourge (71; 1·0 %); *P. granulatum* Bainier (22; 0·37 %); *P. Niklewskii* Zaleski (26; 0·37 %); *P. Raciborskii* Zaleski (22; 0·08 %); unidentified *Penicillium* from soil (33; 1·90 %); *Clasterosporium* sp. (83; 0·0 %); *Mucor pyriformis* Fischer (22; 0·56 %); *Stemphylium* sp. (47; 0·47 %).

Group V. *Metabolized 5–6 % of the total chloride originally present.* 16 species = 12 % of all species examined. *Aspergillus fumigatus* Fresenius (strain No. 47) (47; 0·35 %); *A. nidulans* (Eidam) Winter (46; 0·05 %); *A. ochraceus* Wilhelm (47; 0·24 %); *A. sydowi* (Bainier and Sartory) Thom and Church (46; 0·0 %); *Penicillium biforme* Thom (19; 0·05 %); *P. chrysogenum* Thom (strain P. 57) (25; 0·0 %); *P. citreo-roseum* Dierckx (strain P. 22) (23; 0·45 %); *P. fellutanum* Biourge (strain P. 60) (84; 0·0 %); *P. frequentans* Westling (strain P. 80) (70; 0·0 %); *P. griseo-fulvum* Dierckx (strain P. 68) (70; 0·0 %); *P. Hagemi* Zaleski (68; 0·0 %); *P. ochraceum* (Bainier) Thom (68; 0·0 %); *P. roqueforti* Thom (strain P. 93) (69; 0·26 %); *P. Siemaszki* Zaleski (69; 0·05 %); *P. spinulosum* Thom (25; 0·05 %); *Stysanus stemonites* (Persoon) Corda (48; 0·13 %).

Group VI. *Metabolized 6–7 % of the total chloride originally present.* 14 species = 10 % of all species examined. *Aspergillus fumigatus* Fresenius (strain H.A. 1) (56; 0·46 %); *A. terreus* Thom (strain Ac. 107) (60; 0·0 %); *A. Wentii* Wehmer (46; 0·58 %); *Penicillium baculatum* Westling (62; 0·58 %); *P. baiiolum* Biourge (67; 0·0 %); *P. bialowiezense* Zaleski (53; 0·0 %); *P. caseicolum* Bainier (64; 0·0 %); *P. citreo-roseum* Dierckx (strain P. 23) (25; 0·37 %); *P. citrinum* Thom (strain P. 26) (60; 0·0 %); *P. cyaneo-fulvum* Biourge (strain P. 31) (21; 1·57 %); *P. lividum* Westling (33; 0·01 %); *P. solitum* Westling (39; 0·08 %); *P. subcinereum* Westling (strain P. 98) (69; 0·08 %); *P. sublateritium* Biourge (42; 0·0 %).

Group VII. *Metabolized 7–10 % of the total chloride originally present.* 11 species = 8 % of all species examined. *Aspergillus flavipes* (Bainier and Sartory) Thom and Church (56; 0·02 %); *Penicillium aurantio-brunneum* Dierckx (80; 0·0 %); *P. brunneo-rubrum* Dierckx (strain P. 8) (19; 0·21 %); *P. carmino-violaceum* Dierckx (71; 0·0 %); *P. flavo-cinereum* Biourge (70; 0·08 %); *P.*

frequentans Westling (strain P. 82) (70; 0·05 %); *P. fusco-glaucum* Biourge (21; 0·47 %); *P. Paczoskii* Zaleski (71; 0·08 %); *P. roqueforti* Thom (strain P. 83) (69; 0·21 %); *P. roseo-citreum* Biourge (25; 1·36 %); *Botrytis cinerea* Persoon (28; 1·46 %).

Group VIII. *Metabolized 10–15 % of the total chloride originally present.* 23 species = 17 % of all species examined. *Aspergillus terreus* Thom (strain No. 3) (62; 0·0 %); *A. terreus* (strain No. 37) (62; 0·0 %); *A. terreus* (strain Ab. 2) (60; 0·0 %); *Penicillium chlorophaeum* Biourge (22; 0·0 %); *P. chrysogenum* Thom (strain P. 17) (22; 0·0 %); *P. chrysogenum* (strain P. 19) (23; 0·39 %); *P. chrysogenum* (strain H.P. 1) (22; 0·55 %); *P. citreo-sulfuratum* Biourge (strain P. 66) (71; 0·0 %); *P. citrinum* Thom (strain P. 6) (80; 0·0 %); *P. citrinum* (strain P. 27) (21; 0·50 %); *P. Daleae* Zaleski (68; 0·0 %); *P. flavi-dorsum* Biourge (70; 0·0 %); *P. frequentans* Westling (strain P. 28) (78; 0·0 %); *P. griseo-brunneum* Dierckx (22; 0·13 %); *P. meleagrinum* Biourge (23; 3·38 %); *P. minio-luteum* Dierckx (77; 0·13 %); *P. obscurum* Biourge (68; 0·0 %); *P. Oledzkii* Zaleski (69; 0·08 %); *P. phaeo-janthinellum* Biourge (22; 0·05 %); *P. Raistrickii* G. Smith (53; 0·02 %); *P. rugulosum* Thom (22; 0·0 %); *Cladosporium herbarum* (Persoon) Link (69; 0·0 %); *Caldariomyces fumago* Woronichin (strain X. 14) (41; 2·28 %).

Group IX. *Metabolized 15–25 % of the total chloride originally present.* 13 species = 9 % of all species examined. *Aspergillus itaconicus* Kinoshita (24; 0·42 %); *A. niger* van Tieghem (strain Ac. 1) (21; 0·34 %); *A. terreus* Thom (strain Am. 1) (77; 0·0 %); *A. terreus* (strain Ab. 1) (18; 0·75 %); *A. versicolor* (Vuillemin) Tiraboschi (strain No. 35) (35; 0·85 %); *Penicillium candido-fulvum* Dierckx (20; 2·66 %); *P. chloro-leucon* Biourge (22; 0·32 %); *P. commune* Thom (21; 0·45 %); *P. griseo-roseum* Dierckx (22; 0·71 %); *P. Westlingi* Zaleski (70; 0·0 %); *Alternaria tenuis* Nees (35; 0·85 %); *Syncephalastrum racemosum* (Cohn) Schröter (74; 2·33 %); *Trichoderma viride* Persoon ex Fries (74; 1·57 %).

Group X. *Metabolized over 25 % of the total chloride originally present.* 5 species = less than 4 % of all species examined. *Absidia spinosa* Lendner, 26·6 % of chloride metabolized (74; 0·0 %); *Aspergillus terreus* Thom (strain Am. 2), 33·5 % of chloride metabolized (77; 0·0 %); *A. terreus* (strain No. 45), 96·8 % of chloride metabolized (38; 0·13 %); *Caldariomyces fumago* Woronichin (strain X. 13), 71·5 % of chloride metabolized (33; 1·5 %); *Caldariomyces fumago* (strain Ag. 92), 95·7 % of chloride metabolized (46; 1·47 %).

These results indicate that extensive utilization of chlorides by the lower fungi is of very rare occurrence. Thus of 139 species and strains in a number of different genera by far the greater number utilized less than 10 %, and only five utilized more than a quarter, of the chloride present in the medium.

Cl balance sheet for Aspergillus terreus *Thom, strain No. 45*

The course of Cl metabolism by *A. terreus*, strain No. 45, has already been examined by Raistrick & Smith [1936] but no complete Cl balance sheet for this organism has been reported previously. In order to prove whether geodin and erdin are the only Cl-containing metabolic products formed by this strain the following experiment was carried out. 100 one-litre flasks each containing 350 ml. of the usual Czapek-Dox medium (see p. 667) were sown with *A. terreus*, strain No. 45, incubated at 24° and examined periodically for residual glucose (by polarimeter) and residual chloride (by the Volhard method). After 26 days, when the residual glucose was 0·48 % the contents of 89 flasks were filtered, the mycelium carefully washed and dried and the washings combined with the filtrate.

In a portion of the measured filtrate and washings there were estimated:

(I) Residual inorganic chloride (see p. 667).

(II) Total Cl in solution. An aliquot was evaporated to dryness in a nickel crucible, fused with KOH (10 g.) and Na_2O_2 (5 g.), and the inorganic chloride was estimated by the Volhard method.

(III) Organic Cl in solution. Obtained by subtracting I from II.

To the main bulk of the solution 2*N* H_2SO_4 (825 ml.) was added and the flocculent precipitate formed was separated by filtration after standing overnight. Cl estimations were then carried out on

(IV) The dried H_2SO_4 precipitate (44·45 g.). By fusion with KOH and Na_2O_2 as in II.

(V) The filtrate from IV. As in IV.

(VI) The dried mycelium (307 g.). As in IV.

The results obtained are given in Table I.

Table I

Fraction	Cl as KCl g.	Cl as % of KCl originally present
Original metabolism solution	15·575	100·0
(I) Residual inorganic chloride	0·460	2·95
(II) Total Cl in solution	13·809	88·65
(III) Organic Cl in solution	13·349	85·70
(IV) Cl in H_2SO_4 precipitate	9·672	62·10
(V) Total Cl in acid filtrate	4·019	25·80
(VI) Cl in mycelium	1·835	11·78

Sum of (II) and (VI) equals 100·43 %.

Ether extraction of the dried acid precipitate (IV) led to the isolation of considerable amounts of geodin and erdin but no other Cl-containing compounds could be detected.

Ether extraction of the acid filtrate (V) removed the whole of the organic Cl in solution very readily and from the evaporated ether solution a little over 3 g. of erdin and a small amount of geodin were obtained but no other Cl-containing substances could be isolated.

The mycelium (VI) containing 11·78 % of the Cl originally present in the medium was extracted with light petroleum, B.P. 50–60°, and then with ether but no geodin or erdin was obtained nor could any Cl be detected in any form in either extract. The form in which the Cl is contained in the mycelium must await future investigation.

Part B. Cl metabolism of Caldariomyces fumago *Woronichin. Isolation, properties and molecular constitution of caldariomycin,* $C_5H_8O_2Cl_2$

The two cultures used were:

(*a*) *Caldariomyces fumago*, strain Ag 92. Obtained from Centraal-bureau voor Schimmelcultures, Baarn, Holland, in 1925. Some of the metabolic products of this strain, none of which contained Cl, have already been described by Birkinshaw & Raistrick [1931].

(*b*) *Caldariomyces fumago*, strain X. 13. Obtained from Baarn in 1937.

Caldariomyces fumago Woronichin was formerly known as *Fumago vagans* Persoon and was given its present name by Woronichin [1926]. Van Beyma thoe Kingma [1931] states that it is of fairly common occurrence as sooty mould of greenhouse plants in Northern Europe.

LINDA HALL LIBRARY
KANSAS CITY, MO.

Course of Cl metabolism by Caldariomyces fumago, *strain Ag 92.* The figures given in Table 2 indicate the course of Cl metabolism by *Caldariomyces fumago*, strain Ag 92, when grown at 24° on (*a*) Czapek-Dox 5% glucose solution (see p. 667) containing the usual amount of KCl, i.e. 0·5 g. per litre (see first five items in Table), and (*b*) Czapek-Dox 5% glucose solution containing double the usual amount of KCl, i.e. 1·0 g. per litre (see second five items in Table). All weights are expressed as g. per flask containing 350 ml. of medium.

Table 2

Incubation period days	Residual glucose by polarimeter %	Mycelium (dry wt.) g.	Total Cl in solution calc. as KCl g.	Cl in solution as inorganic chloride calc. as KCl g.	Cl in solution in organic combination calc. as KCl g.	Cl in solution in organic combination calc. as % of KCl originally present %
0	5·00	—	0·1750	0·1750	—	—
14	3·43	—	0·1731	0·0992	0·0739	42·2
18	2·47	3·78	0·1675	0·0627	0·1048	59·9
29	1·18	—	0·1498	0·0084	0·1414	80·8
37	1·62	—	0·1091	0·0082	0·1009	57·6
0	5·00	—	0·3500	0·3500	—	—
14	3·31	2·40	0·3215	0·2013	0·1202	34·3
29	1·08	6·10	0·2050	0·0522	0·1528	43·6
35	1·10	6·10	0·2610	0·0615	0·1995	57·0
42	1·28	5·30	0·2330	0·0746	0·1584	45·3

Investigation of the metabolic products of Caldariomyces fumago. One hundred 1-litre conical flasks, each containing 350 ml. of Czapek-Dox solution having preferably double the usual amount of KCl, i.e. 1 g. per litre instead of 0·5 g., were sterilized, sown with a spore suspension of *Caldariomyces fumago*, strain Ag 92, and incubated at 24° for about 35 days. The strongly acidic contents of the flasks (residual glucose, 1·3–1·5%) were filtered and the mycelium was washed and dried. The mycelium washings were added to the main filtrate, evaporated *in vacuo* at 40–45° to about 1 litre, and exhaustively extracted with ether. Estimation of the total Cl and Cl as residual KCl in solution showed that ether extraction removes the whole of the Cl present in organic combination.

The ether solution, dried over anhydrous $MgSO_4$, was concentrated to a small volume and was then allowed to evaporate to dryness spontaneously in air. There remained a mixture of a yellowish-brown oil and colourless crystalline material. The pasty mass was pressed on a porous plate and the solid material remaining on the plate was boiled with chloroform (10 ml. per 1 g. of total solid) and filtered. On cooling the chloroform solution practically pure *Caldariomycin* separated as large colourless needles, M.P. 120°.

The solid material insoluble in chloroform proved to be fumaric acid. It was purified by sublimation in a high vacuum at 110–120° and was obtained as a colourless, microcrystalline sublimate, M.P. 290° (in a sealed tube), alone or in admixture with authentic fumaric acid. (Found: C, 41·41; H, 3·62; equiv. by titration, 59·1. $C_4H_4O_4$ requires C, 41·36; H, 3·47%; equiv., as a dibasic acid, 58·0.)

A little more caldariomycin was obtained by extraction with ether of the porous tile containing the yellowish-brown oil and repeating the process described above. An oil remained finally from which nothing crystalline has been separated but which contains considerable amounts of Cl in organic combination.

Since caldariomycin contains Cl the use of chloroform as a solvent is open to an obvious theoretical objection and it became necessary to prove that the Cl in caldariomycin does not arise from the solvent used. This was readily done by both the following methods. (*a*) In a second preparation a portion of the crude tiled mixture of metabolic products direct from the ether extraction and without treatment with chloroform was sublimed in a high vacuum at 60°. The colourless crystalline sublimate containing 40·7 % Cl melted at 120–121° alone or in admixture with caldariomycin prepared by crystallization from chloroform. The residue in the sublimation tube consisted of crude fumaric acid. (*b*) A second portion was extracted with boiling light petroleum, B.P. 40–50°, 15 ml. per 1 g. Fumaric acid remained undissolved and caldariomycin separated from the filtered hot light petroleum solution on cooling, M.P. and mixed M.P. 121°. Chloroform is however a much better solvent for the crystallization of caldariomycin than is light petroleum.

Addition of 4 vol. of alcohol to the ether-extracted metabolism solution precipitated a crude polysaccharide which was dried by grinding with absolute alcohol and then with ether. There were thus obtained 285 g. of a greyish-brown powder (ash, 13·7 %), obviously mainly polysaccharide in nature since it gives large amounts of reducing sugars on acid hydrolysis and is essentially the same polysaccharide as was isolated from this strain of *Caldariomyces fumago* by Birkinshaw & Raistrick [1931].

In three experiments with *Caldariomyces fumago*, strain Ag 92, in which the Czapek-Dox solution contained 0·5 g. KCl per litre in exp. 1 and 1·0 g. KCl per litre in exps. 2 and 3, the following yields of metabolic products were isolated.

	Pure caldariomycin g.	Fumaric acid g.	Uncrystallizable oil g.
Exp. 1	4·3	2·1	11·6
Exp. 2	6·1	2·5	14·4
Exp. 3	6·3	3·5	16·95

Since the uncrystallizable oil has a considerable Cl content it is obvious that a large proportion of the Cl metabolized by this strain is present as metabolic products which have not yet been characterized.

With *Caldariomyces fumago*, strain X. 13, 100 flasks of Czapek-Dox solution containing 0·5 g. of KCl per litre gave only 0·4 g. of pure caldariomycin, traces of fumaric acid and 15·6 g. of uncrystallizable oil.

A Cl balance sheet was prepared for *Caldariomyces fumago*, strain Ag 92, grown on Czapek-Dox solution containing 0·5 g. KCl per litre. In this experiment 73 flasks were incubated at 24° for 37 days. At the end of the incubation period the average residual glucose was 0·53 %, the total mycelium weighed 373 g. and the mixed metabolism solution had the analysis given in Table 3. There were isolated from the evaporated mixed metabolism solution 3·10 g. of pure caldariomycin, 2·65 g. of fumaric acid and 7·35 g. of uncrystallizable oil. It will be seen from Table 3 that rather less than one-third of the organic Cl in solution (III) was recovered as pure caldariomycin (V).

It will be seen that the mycelium of *Caldariomyces fumago*, strain Ag 92, contains over 20 % of the Cl originally present in the medium as KCl. All attempts to isolate any product from the dried mycelium which would account for this Cl-containing constituent have so far been unsuccessful. Extraction of the dried mycelium successively with light petroleum (B.P. 40–50°), ether and methanol removed about 13 % of material which however did not contain any

Table 3

Fraction	Cl as KCl g.	Cl as % of KCl originally present
Original metabolism solution	12·775	100·0
(I) Total Cl in solution	9·7073	75·99
(II) Residual inorganic chloride	0·3087	2·42
(III) Organic Cl in solution	9·3987	73·57
(IV) Total Cl in mycelium	2·9280	22·92
(V) Cl in caldariomycin (3·1 g.)	2·705	21·17

Sum of (I) and (IV) equals 98·91%.

Cl. Extraction with cold saturated $NaHCO_3$ solution removed about 35% and with $N/10$ NaOH about 71% of the total Cl in the mycelium, while boiling for 15 hr. with 33% H_2SO_4 removed 85%.

General properties of caldariomycin. Caldariomycin, $C_5H_8O_2Cl_2$, forms fine, colourless needles, M.P. 121°, without obvious decomposition. (Found: C, 35·19, 35·00; H, 4·73, 4·62; Cl, 41·2, 41·4%; CH_3O, nil; N, nil; mol. wt., cryoscopic in dioxane, 158, cryoscopic in nitrobenzene, 197. $C_5H_8O_2Cl_2$ requires C, 35·09; H, 4·71; Cl, 41·5%; mol. wt. 171.) Optical rotation $[\alpha]_{5461}^{20°}+59·2°$ ($c=0·338$ in water).

A Zerewitinoff determination carried out in anisole at room temperature indicated the presence of 1·94 active H atoms per mol.

A Kuhn-Roth chromic acid oxidation gave only 0·2 mol. of acetic acid per mol. of caldariomycin, thus indicating the absence of CH_3 side-chains.

Caldariomycin is readily soluble in cold water, ethanol, ether and in most other organic solvents with the exception of light petroleum and chloroform, in which however it is still appreciably soluble at 0°. Its aqueous solution is neutral in reaction, does not give any colour with $FeCl_3$, or any reaction with Schiff's or Brady's reagents, nor does it decolorize aqueous $KMnO_4$. It reduces Fehling's solution in the cold but only after standing for several minutes, owing probably to the formation in the strongly alkaline solution of some degradation product (see below). On warming solid caldariomycin with conc. H_2SO_4 a reddish-brown solution, with an intense green fluorescence, is obtained.

The two Cl atoms in its molecule are particularly labile. Although they are almost unaffected by treatment with cold saturated $NaHCO_3$ solution they are almost completely liberated as chloride on keeping a cold, dilute solution in $N/10$ NaOH overnight. This alkaline solution, after acidification, now gives a precipitate with Brady's reagent.

No crystalline product was obtained when caldariomycin was treated with phenyl *iso*cyanate under the usual conditions for the formation of a urethane. Similarly all attempts to replace the Cl atoms by other groups failed to yield crystalline products. These included refluxing with a solution of silver acetate or potassium acetate in methanol (no action); treatment with KCN under conditions which convert 2-chloro*cyclo*hexanone into 2-cyano*cyclo*hexanone [Mayer, 1933]; and an attempt to condense caldariomycin with the sodium derivative of ethyl malonate.

Caldariomycin gives a good reddish purple colour with alcoholic *m*-dinitrobenzene and alcoholic KOH under the conditions for the modified Zimmermann reaction for active CH_2 groups laid down by Callow *et al.* [1938]. The following experiment shows however that it is not caldariomycin itself which contains the active CH_2 group but a product formed from it by the action of the KOH. An alcoholic solution of caldariomycin (1 ml. containing a few mg.) was mixed with

18% alcoholic KOH (4 drops) and left for 20 min. at room temperature; 2% alcoholic *m*-dinitrobenzene (1 ml.) was then added and the coloration became perceptible in 10 sec. and very intense in 1 min. In a control experiment, using the same alcoholic solution of caldariomycin (1 ml.) but adding the alcoholic KOH and *m*-dinitrobenzene together, the colour was only just perceptible in 1 min. and not very pronounced after 3 min.

Action of heat on caldariomycin. Caldariomycin (3 g.) was heated in a slow stream of N_2 in a boiling tube connected to two bubblers containing a solution of 2:4-dinitrophenylhydrazine in 2*N* HCl. Ebullition of the melt began at 185° and proceeded vigorously at 195–200° at which temperature the oil bath was maintained for 30 min. when ebullition ceased. Water and streams of HCl gas were evolved and the bulk of the caldariomycin was converted into a black, non-volatile, viscous resin. A small amount of a colourless, mobile liquid passed over into the bubblers and gave an immediate orange-red precipitate in the Brady's solution. This precipitate was collected at once (0·10 g.) and the filtrate, on standing overnight, deposited a further amount of a very similar product (0·20 g.). By fractional crystallization from ethanol two dinitrophenylhydrazones were isolated. The less sparingly soluble one consisted of light orange-red rods, M.P. 238° (decomp.), whilst the more sparingly soluble constituent formed very dark red irregular crystalline aggregates, M.P. 224° (decomp.). (Found: (*a*) On the dinitrophenylhydrazone, M.P. 238°, C, 44·86; H, 3·22; Cl, 11·1%. (*b*) On the dinitrophenylhydrazone, M.P. 224°, C, 44·98, 45·07; H, 3·22, 3·27; N, 18·7; Cl, 10·8%. $C_{11}H_9O_4N_4Cl$, i.e. the mono-dinitrophenylhydrazone of C_5H_5OCl, requires C, 44·51; H, 3·06; N, 18·9; Cl, 12·0%.) The two compounds are therefore isomeric and since they both gave a red coloration with alcoholic NaOH they are both mono-dinitrophenylhydrazones.

The mono-dinitrophenylhydrazone, M.P. 224°, was also isolated in small yield from the products of the acid hydrolysis of caldariomycin. A solution of caldariomycin (0·5 g.) in 2*N* H_2SO_4 (50 ml.) was boiled for 2 hr. in an atmosphere of N_2. At the end of this time 15·3% of the Cl in the caldariomycin had been converted into HCl. There was isolated from the hydrolysis products a small amount of an orange dinitrophenylhydrazone which was purified by crystallization, first from ethanol, then from xylene and finally from a large volume of ethanol. It formed small red needles, M.P. 226° (decomp.), which gave the red colour with alcoholic NaOH typical of a mono-dinitrophenylhydrazone. (Found: C, 44·23, 44·28; H, 2·97, 3·20; N, 19·6; Cl, 10·4%. $C_{11}H_9O_4N_4Cl$ requires C, 44·51; H, 3·06; N, 18·9; Cl, 12·0%.)

Proof that caldariomycin does not contain the grouping $\rangle C.(OH).C.(OH)\langle$. Malaprade [1928] and Fleury & Lange [1932; 1933] have shown that polyhydric alcohols containing hydroxyl groups attached to adjacent carbon atoms react with periodic acid with the formation of aldehydes. Malaprade [1934] and Clutterbuck & Reuter [1935] have also shown that α-ketols, α-diketones and α-ketonealdehydes are readily oxidized by this reagent. Since caldariomycin is unaffected by HIO_4 while the two forms of *cyclo*pentane-1:2-diol are both oxidized it is clear that caldariomycin does not contain the grouping

$$\rangle C.(OH).C(OH)\langle .$$

(*a*) A solution of caldariomycin (0·1122 g.) in 0·3% KIO_4 solution (100 ml.) was acidified with 2*N* H_2SO_4 (10 ml.) and kept overnight side by side with a blank experiment. Excess KI was then added to each flask and the liberated iodine was titrated with *N*/10 $Na_2S_2O_3$. The experimental flask required 106·7 ml. and the blank 106·8 ml. thus proving that no oxidation had taken place.

(*b*) The lower-melting *cyclo*pentane-1:2:diol (M.P. 10°; 0·234 g. [Godchot & Taboury, 1912, 1913]) when oxidized as above utilized oxygen equivalent to 18·4 ml. *N*/10 iodine which corresponds to about 40 % of complete oxidation of

.CHOH.CHOH. to .CHO + .CHO.

(*c*) The higher-melting *cyclo*pentane-1:2-diol (M.P. 46–48°; 0·117 g. [Meiser, 1899]) when oxidized similarly utilized oxygen equivalent to 22·4 ml. *N*/10 iodine corresponding to 97·7 % complete oxidation.

Oxidation of caldariomycin with chromic acid. Formation of succinic acid. To a solution of caldariomycin (0·344 g.) in 2*N* H_2SO_4 (30 ml.) was added a solution of CrO_3 (0·54 g.) in 2*N* H_2SO_4 (20 ml.). The mixture was kept at room temperature for 7 days and was then extracted with ether. The ether extract was evaporated to dryness, the residue (0·225 g.) titrated with *N*/10 NaOH to phenolphthalein and re-extracted with ether to remove some unchanged caldariomycin. The aqueous layer was then acidified, extracted with ether, the solvent removed and the crystalline residue was purified by sublimation in a high vacuum to give a colourless crystalline sublimate (0·1 g.) of succinic acid, M.P. 186°, alone or in admixture with an authentic specimen of succinic acid. (Found: equiv. by titration, 64·0. $C_4H_6O_4$, titrating as a dibasic acid requires equiv. 59·0.)

Catalytic reduction of caldariomycin. Formation of cyclo*pentanone.* When caldariomycin is reduced with hydrogen, using a palladium-charcoal catalyst, uptake of H_2 is slow throughout the reduction which appears to indicate the absence of a double bond from caldariomycin. Cl is removed from the molecule and appears as HCl in the solution.

Caldariomycin (1·0 g.) dissolved in water (50 ml.) was shaken for 8 hr. with a catalyst prepared from $PdCl_2$ (1 g.) and norite (3 g.) in an atmosphere of H_2 until no further absorption of H_2 took place. The volume of H_2 absorbed was about 80 % of that required by the equation,

$$C_5H_8O_2Cl_2 + 2H_2 \rightarrow C_5H_8O + H_2O + 2HCl.$$

Excess of Brady's reagent was added to the filtered liquid and the crude Cl-free dinitrophenylhydrazone which was precipitated was collected after several hours, washed and dried (0·25 g., M.P. about 140°). It formed long orange needles from ethanol, M.P. 144°, alone or in admixture with an authentic specimen of *cyclo*pentanone dinitrophenylhydrazone (M.P. 144°; lit. 142°) prepared from *cyclo*pentanone [Gilman, 1932] and recrystallized similarly. The two specimens had the same crystalline form, and both gave with alcoholic NaOH the same red colour typical of a mono-dinitrophenylhydrazone. (Found: on dinitrophenylhydrazone of reduction product, C, 50·25; H, 4·39; N, 22·4 %. $C_{11}H_{12}O_4N_4$, i.e. the mono-dinitrophenylhydrazone of C_5H_8O, requires C, 49·98; H, 4·58; N, 21·2 %.)

The yield of *cyclo*pentanone dinitrophenylhydrazone obtained obviously accounts for only a fraction of the caldariomycin used. In a second experiment 4 g. of caldariomycin were reduced; the mixture was filtered, neutralized with *N* NaOH and evaporated to dryness under reduced pressure at 40°. 1·12 g. of *cyclo*pentanone dinitrophenylhydrazone, M.P. 140–142°, were obtained from the distillate after treatment with excess of Brady's reagent. The residue remaining after evaporation was extracted with methanol. On removal of the solvent there remained a solid mixed with some oily material from which the only pure product that could be isolated was unchanged caldariomycin.

Since neither of the *cyclo*pentane-1:2-diols (see above) gives an appreciable precipitate with Brady's reagent, even on long standing, and since neither of

them would distil under the conditions described, it is clear that the primary reduction product of caldariomycin cannot be a *cyclo*pentane-1:2-diol. It appears therefore that *cyclo*pentanone is itself a primary reduction product of caldariomycin and does not arise by dehydration of some intermediate dihydroxy compound through the action of the HCl in Brady's reagent.

Br metabolism of Caldariomyces fumago, *strain Ag 92.* When *Caldariomyces fumago*, strain Ag 92, was sown on the ordinary Czapek-Dox glucose solution containing equivalent amounts of KBr or KI instead of the usual 0·5 g. per litre of KCl no growth was obtained on the KI medium though fairly normal growth resulted on the KBr medium with the formation of metabolic products containing bromine in organic combination which, however, have not yet been characterized.

100 flasks each containing 350 ml. of Czapek-Dox 5% glucose solution (see p. 667) with 29 g. of KBr per 35 litres in place of 17·5 g. KCl were inoculated with *Caldariomyces fumago*, strain Ag 92, and incubated at 24°. At intervals single flasks were selected and the following estimations were carried out on each flask. (*a*) Residual glucose by polarimeter. (*b*) Total Br in solution. (*c*) Br in solution as inorganic bromide. (*d*) Br in solution in organic combination, i.e. (*b*)–(*c*). (*e*) Weight of mycelium. The results obtained are given in Table 4 in which all weights are expressed as g. per flask. Growth was very slow during the first 7 days but after about 10 days a thin mycelial felt was established and growth then became almost normal with the ultimate formation of a thick brownish-black compact felt. The metabolism solution gradually became reddish brown in colour and viscous owing to the formation of a polysaccharide which interfered with the Br estimations. This difficulty was overcome by precipitating the polysaccharide with 4 vol. of alcohol. The Br in solution was then estimated by the usual volumetric and gravimetric methods on the polysaccharide-free solution after removal of the alcohol.

Table 4

Incubation period days	Residual glucose by polarimeter %	Mycelium (dry wt.) g.	Total Br in solution calc. as KBr g.	Br in solution as inorganic bromide calc. as KBr g.	Br in solution in organic combination calc. as KBr g.	Br in solution in organic combination calc. as % of KBr originally present %
0	5·00	—	0·290	0·290	—	—
14	4·83	0·62	0·290	0·244	0·046	15·9
21	4·36	—	0·279	0·200	0·079	27·2
28	3·00	3·75	0·248	0·163	0·085	29·3
35	2·70	4·75	0·279	0·149	0·130	44·8
38 (average of 96 flasks)	1·02	3·77*	0·244	0·136	0·108	37·2

* The Br content of the mycelium ≡ 0·026 g. KBr per flask.

After 38 days' incubation the metabolism solution from the 96 flasks remaining was filtered, evaporated *in vacuo* at 40° to about 1 litre and then exhaustively extracted with ether. The reddish-brown ethereal extract, on removal of the solvent, gave 11 g. of a dark reddish-brown oil containing a considerable proportion of Br. So far the only pure substance isolated from this oil is fumaric acid. The investigation of this somewhat intractable material is being continued in the hope of isolating from it in a state of purity the Br-containing metabolic product which is obviously present.

SUMMARY

A quantitative survey has been made of the chlorine metabolism of 139 species or strains of moulds grown on Czapek-Dox 5% glucose solution containing 0·5 g. per litre of KCl as the sole source of chlorine. Extensive conversion of inorganic chloride into organic chlorine-containing metabolic products is of rather rare occurrence, though with a number of species this conversion is by no means negligible. The chlorine metabolism of one of the species which is most active in this respect—*Caldariomyces fumago* Woronichin—has been investigated in detail and has resulted in the isolation of a hitherto undescribed mould metabolic product, *caldariomycin*, $C_5H_8O_2Cl_2$, which has been shown to be probably *2:2-dichlorocyclopentane-1:3-diol*. The same species also converts KBr, when present in the same medium in place of KCl, into organic bromine-containing metabolic products which have not yet been isolated in a state of purity.

We wish to thank our colleague Mr G. Smith for much technical help in the preparation of cultures.

REFERENCES

Van Beyma thoe Kingma (1931). *Verh. Akad. Wet. Amst.* Sect. 2, **29**, 2, pp. 1–29.
Birkinshaw & Raistrick (1931). *Philos. Trans.* B, **220**, 341.
Calam, Clutterbuck, Oxford & Raistrick (1939). *Biochem. J.* **33**, 579.
Callow, Callow & Emmens (1938). *Biochem. J.* **32**, 1312.
Clutterbuck, Koerber & Raistrick (1937). *Biochem. J.* **31**, 1089.
—— & Reuter (1935). *J. chem. Soc.* p. 1467.
Fleury & Lange (1932). *C.R. Acad. Sci. Paris*, **195**, 1395.
—— —— (1933). *J. Pharm. chim. Paris*, **17**, 409.
Gilman (1932). Organic syntheses, Collective Vol. **1**, 187. John Wiley and Sons.
Godchot & Taboury (1912). *C.R. Acad. Sci. Paris*, **154**, 1625.
—— —— (1913). *Bull. Soc. chim. Paris*, (4), **13**, 536.
Levy & Jedlicka (1888). *Liebigs Ann.* **249**, 89.
Malaprade (1928). *C.R. Acad. Sci. Paris*, **186**, 382.
—— (1934). *Bull. Soc. chim. Paris*, (5), **1**, 833.
Mayer (1933). *Helv. chim. Acta*, **16**, 1291.
Meiser (1899). *Ber. dtsch. chem. Ges.* **32**, 2050.
Oxford, Raistrick & Simonart (1939). *Biochem. J.* **33**, 240.
Raistrick & Smith (1936). *Biochem. J.* **30**, 1315.
Woronichin (1926). *Ann. mycol., Berl.*, **24**, 261.
Zincke & Kegel (1889). *Ber. dtsch. chem. Ges.* **22**, 1474.

82. ELIMINATION OF 3:4-BENZPYRENE FROM THE RAT[1]

By JAMES GOWANS CHALMERS[2]

From The Research Department of The Glasgow Royal Cancer Hospital

(*Received 15 March 1940*)

In a previous communication [Chalmers, 1938] spectrographic evidence was given to show that intravenously injected 3:4-benzpyrene was eliminated via the bile as a fluorescent derivative in the faeces of the mouse. In the present investigation, a further study of the elimination of this carcinogen from the animal body has been made. Rats were used instead of mice to obtain larger quantities of the fluorescent material, and the urine was examined as well as the faeces for fluorescent derivatives of 3:4-benzpyrene. The fluorescence of the extracts made in the course of the investigation was taken as an indication of the presence of elimination products, the initial visual observation in the ultra-violet beam being confirmed, if necessary, by spectrographic analysis.

Experimental

The rats were injected intravenously in the tail vein with a colloidal solution of 3:4-benzpyrene and kept in metabolism cages in which the faeces and urine could be collected separately.

In the first instance, a series of 12 young rats were injected with 1 ml. of 0·03 % colloid on alternate days. This was about the maximum quantity of colloid which could be injected into small rats and, in subsequent experiments, larger animals were used.

In a series of 21 adult rats, an attempt was made to inject each rat with 2–3 ml. of 0·03 % 3:4-benzpyrene colloid on alternate days, but, owing to the difficulty of injecting the animals, occasional irregularities occurred. The quantities of colloid injected are given in Table 1. The maximum amount of hydrocarbon injected into any rat was 39 mg., and 11 of the rats were under examination for 10 months, or more.

Although 3:4-benzpyrene proved to be toxic in the rat, the animals tolerated the quantity injected well and, in certain cases where the administration of the colloid was stopped owing to difficulties in making the injections, the animals survived for 14 months.

It is interesting to note that, in this investigation, the intravenous injection of 3:4-benzpyrene did not produce lung tumours. Previous work [Chalmers, 1938] has shown that after the intravenous injection of a colloidal solution of this carcinogen the lungs of a mouse showed a number of fluorescent spots which proved to be particles of the unchanged hydrocarbon. The same deposition takes place in the rat, and it appears that no lung tumours were found in the experiments described because: (1) the rat lung was not susceptible to the

[1] A preliminary account of this work was submitted to the Third International Cancer Congress, Atlantic City, N.J., U.S.A., September 1939.

[2] Beit Memorial Fellow.

Table 1

No. of Exp.	Rat no.	Time under examination* months	Benzpyrene colloid injected intravenously ml.	Benzpyrene mg.	Time from last injection until death months
1	4 A	18	51	15	14
2	5 A	18	50	15	14
3	1 A	17	130	39	4
4	2 A	16	117	35	3
5	9 A	16	92	28	7
6	10 A	13	96	29	3
7	11 A	13	85	25	4
8	18 A	13	75	23	10
9	17 A	12	75	23	8
10	16 A	10	75	23	5
11	21 A	10	61	18	7
12	12 A	9	99	30	†
13	6 A	7	49	15	3
14	7 A	5	24	7	4
15	3 A	3	25	8	0
16	8 A	3	24	7	2
17	13 A	3	53	15	0
18	14 A	3	62	19	0
19	15 A	3	77	23	0
20	19 A	3	49	15	0
21	20 A	3	61	18	0

* The time under examination was the time from the first injection of colloid until the death of the animal. Some of the rats survived for more than a year after the last injection, and the time of survival did not appear to bear any definite relationship to the quantity of colloid injected.

† Accidentally discarded.

carcinogenic action of 3:4-benzpyrene, or (2) the 3:4-benzpyrene did not come into contact with the tissues of the lung for a sufficiently long time or in high enough concentration.

Although no malignant change was observed, other pathological changes were found in the lungs of the animals. In some cases, there was evidence of congestion, and, in others, there were regions of bronchiectasis, with areas of squamous metaplasia, showing considerable irregular epithelial hyperplasia and marked keratinization. Histological examination of the kidneys of the rats which died or were killed during the investigation revealed in almost all cases some degree of nephritis, the condition varying from simple congestion to acute glomerular nephritis. The toxic action of 3:4-benzpyrene on the kidney must be discounted to some extent by the fact that gelatin, which was used as a protective colloid, caused haematuria in rats on injection even of a 0·05 % solution. Colloidal solutions of 3:4-benzpyrene produced haematuria in the absence of gelatin, so, evidently, the hydrocarbon had some toxic effect. Post-mortem examination of the spleen in the experiments described did not show any significant pathological change.

Changes in the liver were not so marked as in the kidney or lung, although, presumably, the chemical change of the hydrocarbon takes place chiefly in this organ. In view of these observations on the effect of intravenously injected 3:4-benzpyrene on the organism of the rat, a further study of this toxic action is being made.

Fluorescent derivative in faeces

Extraction. The faeces collected after the intravenous injection of 3:4-benzpyrene colloid were extracted with absolute alcohol under continuous refluxing for 4 hr. and, after grinding up with sand, for a further 4 hr. The

alcoholic extracts obtained in this way were pooled until 2–3 l. of highly fluorescent solution had been collected. The combined alcoholic extracts were then dried over Na_2SO_4 and evaporated to dryness in N_2 under reduced pressure. A solution of the residue in 2*N* NaOH was chilled at 0° and filtered to remove fat. Since there was usually a large amount of fat present, this process was repeated several times to obtain as good a separation of the fluorescent material as possible. The combined alkaline extracts were acidified with conc. HCl and extracted with benzene. This benzene solution was dried over Na_2SO_4 and concentrated to a suitable volume for further purification of the fluorescent substance by chromatographic adsorption on aluminium oxide. An estimate of the volume required was made by the colour of the solution, the initial adjustments being made by trial and error.

Chromatographic adsorption. The adsorption tower consisted of a straight glass tube with a constriction near one end to hold a Seitz filter pad in position. When the tube was more than 1·25 cm. in diameter, a Gooch filter plate was used to support the filter pad.

The adsorption of the fluorescent material was carried out in two stages. In the first stage, most of the associated colouring matter was removed from the fluorescent substance, but a second treatment was usually required to ensure a complete separation.

In the first stage, the dark brown, violet-fluorescent benzene solution was filtered through a $25 \times 2{\cdot}25$ cm. tower of alumina. A 10–15 cm. grey zone, with a violet fluorescence, and bounded below by a dark brown ring, was formed. On development of the chromatogram with ether, a yellow zone with a yellow-green fluorescence was formed under the green zone. The yellow zone was then detached from the rest of the column and eluted with a benzene + methyl alcohol mixture.

The eluate of the yellow zone was evaporated to dryness in N_2 under reduced pressure, and the residue dissolved in benzene. Then the solution, which was much lighter in colour than the original benzene extract, was filtered through a $20 \times 1{\cdot}25$ cm. adsorption tower of alumina. At the top of the column a 3–5 cm. grey zone with a violet fluorescence formed, and immediately underneath there was a brown ring. On development of the chromatogram with a mixture of benzene + ethyl alcohol, a brown zone descended the column and was washed into the filtrate, leaving a yellow zone with a yellow-green fluorescence on the tower. This yellow zone was then eluted with the benzene + methyl alcohol mixture, giving a solution with a yellow colour and a blue-violet fluorescence, even in daylight.

On occasion, it was possible to combine the two stages of the chromatographic adsorption. After the development of the chromatogram with ether, the upper grey zone, which contained little fluorescent material, was removed with a spatula without disturbing the rest of the column, which was then developed with the benzene + ethyl alcohol mixture. The yellow zone formed was eluted, as before.

An experiment was made with normal rat faeces, which were extracted in a similar manner to the fluorescent faeces, but the resultant chromatogram did not show a yellow-green fluorescent zone.

Fluorescence spectrum. The apparatus used for photographing the fluorescence spectrum was the same as that already described [Chalmers & Peacock, 1936]. An alcoholic solution from the eluate of the yellow zone showed fluorescence spectrum bands at (4280–4430) and (4570–4750) Å. As a result of the present investigation, it appears that the fluorescent elimination product of 3:4-benzpyrene found in the faeces of the rat consists of one fraction, showing

the fluorescence spectrum bands recorded, and not two fractions, as described in the preliminary experiments for mice [Chalmers, 1938]. The preliminary experiments were made with the relatively small amounts of fluorescent material obtained from mouse faeces, and the concentrates examined spectrographically were not so highly purified as those which have now been prepared. It seems probable, although the derivatives have not been isolated, that the same fluorescent substance is eliminated in the faeces of the rat and of the mouse after intravenous injection of 3:4-benzpyrene in these animals.

Absorption spectrum. The absorption spectrum of various alcoholic contents in the fluorescent material have been examined. One of the solutions showed selective absorption in the far ultraviolet region of the spectrum, but this result appears to have been due to the presence of spectroscopically interfering substances, since subsequent examination of other concentrates has shown only general absorption.

Instability to light. A sample of the alcoholic concentrate of the fluorescent substance derived from rat faeces was sealed in a 20 cm. length of capillary tubing in the absence of O_2 and then exposed to bright sunlight for a few hours. At the end of this time the intensity of fluorescence was compared with that of a control kept in the dark, and there was a significant reduction in the intensity of the former. The fluorescent material was also found to be photo-labile when exposed to the light in the laboratory, but in this case the reduction in the intensity of the fluorescence did not take place so quickly, showing that the instability was directly related to the intensity of light to which the fluorescent substance was exposed.

Stability to the presence of oxygen and to heat. Samples of the fluorescent material were sealed in quill tubing in the presence of O_2 and of N_2, and stored in the dark overnight. No diminution in the intensity of fluorescence of the samples was observed.

A sample of the alcoholic concentrate was sealed in a capillary tube and immersed in a closed metal water bath, which was heated to 100° for 2 hr. There was no diminution in the intensity of fluorescence of this sample, compared with that of an unheated sample stored in the dark.

Fluorescent material from urine

Extraction. In a preliminary experiment, the rat urine collected after the intravenous injection of 3:4-benzpyrene was acidified with HCl and extracted successively with ether, chloroform and amyl alcohol. The extracts were compared with similar extracts of normal rat urine, and it was found that the ether and amyl alcohol extracts were yellow-brown in colour and had a violet fluorescence in the ultraviolet beam not given by normal samples. The chloroform solution, however, did not appear to contain an extract, fluorescent or otherwise, which might have indicated the presence of an elimination product conjugated with mercapturic acid.

Ether-soluble fraction. The urine collected daily was made slightly acid with HCl, and extracted with ether in a continuous extractor for 6 hr. The ether extracts were pooled until about 2 l. had accumulated, and then, after washing with water, the extract was dried over anhydrous Na_2SO_4 and the ether evaporated off in an atmosphere of N_2, under reduced pressure. On acidification with HCl of a solution of the residue in 2*N* NaOH, a dark-coloured precipitate was formed. An attempt was made to isolate the fluorescent material in the precipitate by chromatographic adsorption on alumina, but it appeared to be present only in small quantity.

Amyl alcohol-soluble fraction. After removal of the ether-soluble fraction, the urine, which was still violet-fluorescent, was extracted with amyl alcohol in a separating funnel. The amyl alcohol solution was dried over Na_2SO_4, and evaporated to small volume in N_2 under reduced pressure. Then the fluorescent material was extracted from the amyl alcohol by treatment with 2*N* NaOH and, on acidification of this alkaline solution, a dark-coloured precipitate was formed. The fluorescent material, however, in this fraction also appeared to be present only in small quantity. The alkaline solution gave a positive test for glucuronic acid, with naphthoresorcinol.

Discussion

3:4-Benzpyrene, after intravenous injection into the rat, was eliminated in the faeces as a fluorescent derivative, soluble in NaOH. The derivative had a blue-violet fluorescence in organic solvents, and a yellow-green fluorescence in alkaline solution. An alcoholic solution showed two diffuse fluorescence bands in the blue-violet region of the spectrum. The extraction of the substance was complicated by its photo-lability, and, as far as possible, all operations were carried out in the dark or in diffuse daylight.

The quantity of 3:4-benzpyrene injected in these experiments was about the maximum which could be tolerated by the rat, but the amount of fluorescent elimination products obtained from the urine and faeces was insufficient to isolate. (In a further series of experiments, a 1 % solution of 3:4-benzpyrene in olive oil was injected subcutaneously into rats, and in this way a large amount of the hydrocarbon has been converted into fluorescent derivatives. The fluorescent derivative eliminated in the faeces after subcutaneous injection is similar in fluorescence and in solubility in NaOH to the derivative obtained after intravenous injection. A report of this further investigation will be presented at a later date.)

Evidence was also found of the elimination of benzpyrene as a fluorescent derivative in the urine. This fluorescent substance consisted of two fractions, one soluble in ether, the other in amyl alcohol, but the separate identity of the two fractions has not been established. Both fractions showed a blue-violet fluorescence in organic solvents and were soluble in NaOH with yellow-green fluorescence. The amyl alcohol fraction gave a positive naphthoresorcinol test for glucuronic acid. The fluorescence of the combined extracts of urine was much less intense than that of the extract of faeces, and, although no definite conclusion can be made until the derivatives have been isolated, it appears that the fluorescent substance in the urine is present in much smaller quantity than that in the faeces.

If this is so, then the elimination of 3:4-benzpyrene in the bile, first described by Peacock [1936] and, subsequently, in the faeces, is the chief route of elimination of the fluorescent derivative in the rat. It is, of course, possible that this carcinogen is also converted into a non-fluorescent derivative, which may be eliminated chiefly in the urine. In respect of elimination in the bile, benzpyrene appears to differ from naphthalene. Stekol & Mann [1937] conclude that bile is not the main channel for the excretion of naphthalene in animals, in a study of the excretion of 1-α-naphthalenemercapturic acid in dogs with biliary fistula. Detoxication products of anthracene in rats and rabbits have been isolated from the urine by Boyland & Levi [1935; 1936, 1, 2]. The animals received the hydrocarbon in their diet and there is no record of an examination of the faeces. In experiments carried out in this laboratory [Chalmers & Peacock, 1936; Chalmers, 1938], in which the fluorescence of extracts of bile and of faeces

obtained after intravenous injection of 3:4-benzpyrene, methylcholanthrene and anthracene were examined spectrographically, evidence was given to show that fluorescent derivatives of these hydrocarbons were eliminated via the bile and faeces. As the fluorescence of the extracts from the animals injected with 3:4-benzpyrene was more intense and showed a banded fluorescence spectrum, most of the work so far has been done with this substance. Further investigation, however, has not altered the original hypothesis that the carcinogens 3:4-benzpyrene and methylcholanthrene are eliminated in the same way as the non-carcinogen, anthracene. The detoxication of naphthalene fed to rats [Stekol, 1937–8] and rabbits [Bourne & Young, 1934], and anthracene fed to rats and rabbits [Boyland & Levi, 1936, 2] by conjugation with mercapturic acid has been described. Consequently, it was anticipated that the detoxication of 3:4-benzpyrene would also involve this type of conjugation, but no evidence of such was found although it is possible that the methods used would not have detected the presence of small quantities of mercapturic acid.

White & White [1939] found that the growth of white rats, which was retarded by the addition of methylcholanthrene, benzpyrene and pyrene to the diet, could be restored by dietary supplements of sulphur-containing amino-acids. They concluded that the hydrocarbons caused "a specific deficiency in the sulphur-containing amino-acids, probably by imposing on the organism an increased requirement for organic sulphur in the form of cystine or methionine for detoxication mechanisms". But they also considered another possibility, that these polycyclic hydrocarbons retard cellular metabolism and that the sulphur-containing amino-acids "may be regarded as general cell metabolic stimulants, with consequent effect on the general growth of the organism".

It is possible that the mode of administration of 3:4-benzpyrene has an effect on the course of its alteration in the animal body. The mercapturic acids of the carbocyclic compounds already isolated have all been obtained from the urine of animals fed with the hydrocarbon, while, in the experiments described in this communication, the hydrocarbon has been injected intravenously. An indication of the presence or absence of the mercapturic acid derivative in the detoxication of 3:4-benzpyrene could be found by the quantitative estimation of the detoxication products, other than the mercapturic acid found in the urine and faeces, but the establishment of the identity of these products is a necessary preliminary to such an investigation.

Summary

The elimination of 3:4-benzpyrene by the rat after intravenous injection has been studied. Evidence is presented to show that the hydrocarbon is eliminated as fluorescent derivatives in the urine and faeces.

The extraction of the fluorescent substance in the faeces, and its purification by chromatographic adsorption on aluminium oxide are described. It is photolabile, and soluble in NaOH, with green-yellow fluorescence. An alcoholic solution shows two diffuse fluorescence bands in the blue-violet region of the spectrum.

I am indebted to Dr L. Woodhouse Price for examining the histological sections, and to Mr C. Bern for technical assistance.

REFERENCES

Bourne & Young (1934). *Biochem. J.* **28**, 803.
Boyland & Levi (1935). *Biochem. J.* **29**, 2679.
—— —— (1936, 1). *Biochem. J.* **30**, 728.
—— —— (1936. 2). *Biochem. J.* **30**, 1225.
Chalmers (1938). *Biochem. J.* **32**, 271.
—— & Peacock (1936). *Biochem. J.* **30**, 1242.
Peacock (1936). *Brit. J. exper. Path.* **17**, 164.
Stekol (1937–8). *J. biol. Chem.* **122**, 333.
—— & Mann (1937). *J. biol. Chem.* **117**, 619.
White & White (1939). *J. biol. Chem.* **131**, 149.

83. EXPERIMENTS ON THE BIOGENESIS OF VITAMIN B_1

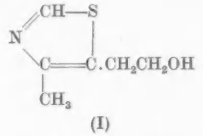
By C. R. HARINGTON and R. C. G. MOGGRIDGE

From the Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

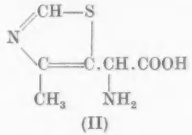
(*Received 21 March 1940*)

We have recently suggested [Harington & Moggridge, 1939; compare also Bonner & Buchman, 1938] that the 4-methyl-5-(β-hydroxyethyl)-thiazole (I) of aneurin might arise *in vivo* from α-amino-β-(4-methylthiazole-5)-propionic acid (II) by a reaction analogous to the formation of "fusel oil" in alcoholic fermentation. The fact that yeast, which is pre-eminently able to effect this type of degradation, is a rich source of aneurin added plausibility to this hypothesis. The synthesis of the amino-acid (II) was described in our last communication. It has now been found that the compound II is in fact broken down by yeast to give 4-methyl-5-(β-hydroxyethyl)-thiazole (I) which was isolated as the picrate; the yield amounted to nearly 60 % on the assumption that only one of the optical isomerides was attacked.

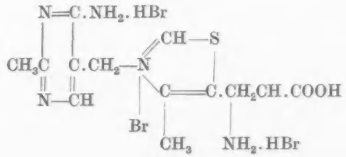
Laevorotatory α-amino-β-(4-methylthiazole-5)-propionic acid of specific rotation $-9{\cdot}0°$ could also be isolated from the fermented liquor: it is thought that this compound is optically pure, since the rotation was not appreciably altered by a second fermentation with yeast. The latter observation is of interest in that it settles the configuration of the optical isomerides of the amino-acid (II): that corresponding to the naturally occurring amino-acids—presumably the one attacked by yeast—must be dextrorotatory while the isomeride isolated will be the $d(-)$-form.



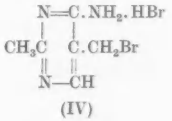
(I)



(II)



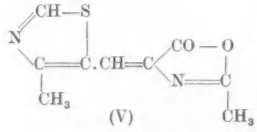
(III)



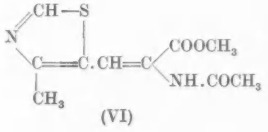
(IV)

It was now decided to try to extend this work by preparing the amino-acid (III) analogous to aneurin. If this compound could be obtained it would be of interest both to see if it could replace aneurin in animal experiments, and to study its fermentation by yeast, with which it might be expected to give both aneurin and cocarboxylase. The work has had to be abandoned for the time being without complete characterization of the compound III: the opportunity is taken here, however, to record the initial stages of the synthesis.

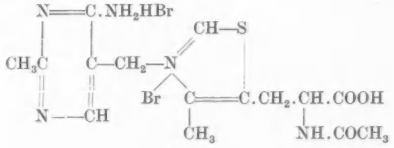
In spite of repeated attempts no conditions could be found in which 2-methyl-4-amino-5-bromomethylpyrimidine hydrobromide (IV) would react with the amino-acid (II). The contrast with the ease of interaction of the pyrimidine (IV) with thiazoles in general suggested that the condensation must in this case be prevented by either the amino- or carboxyl-group in the side chain of II. It was accordingly decided to prepare an ester and an *N*-acylated derivative of II, and to investigate the condensation of these compounds with the pyrimidine (IV). α-Amino-β-(4-methylthiazole-5)-propionic acid methyl ester hydrochloride was obtained without difficulty by the usual method: attempts to prepare the *N*-carbobenzyloxy- or *N*-acetyl-derivatives of this compound by the action of the respective chlorides, however, met with failure. The behaviour of the thiazole amino-acid towards acid chlorides is reminiscent of that of histidine and other glyoxalines, and it seems probable that the acyl chlorides may also attack the basic thiazole ring. An unsuccessful attempt was next made to prepare the *N*-acetylated methyl ester of II from 4-methylthiazole-5-aldehyde. This was condensed with acetylglycine to give the azlactone (V), from which the acetamido-acrylic ester (VI) was easily obtained. No method of reduction of this compound to the required α-acetamido-β-(4-methylthiazole-5)-propionic acid methyl ester could, however, be found.



(V)



(VI)



(VII)

The amino-acid could be acetylated in excellent yield by boiling with acetic acid containing one equivalent of acetic anhydride, and it was found that the condensation of the pyrimidine (IV) with the α-acetamido-β-(4-methylthiazole-5)-propionic acid proceeded smoothly to give the *N*-acetyl derivative (VII) of the required product. This compound was hydrolysed by hydrobromic acid, and a picrate was isolated which analysed as the tripicrate of compound III. III itself has proved hard to purify, and at the stage which the work has reached has not been obtained crystalline in large enough quantities for satisfactory analysis. No claim can therefore be made that the compound is fully characterized.

Experimental

Pressed top yeast (40 g.) was added to α-amino-β-(4-methylthiazole-5)-propionic acid (1 g.) and loaf sugar (80 g.) in water (700 ml.). The mixture was kept for 5 days at room temperature, when fermentation was complete and the yeast was filtered off. The filtrate was acidified with 5*N* HCl (5 ml.) and concentrated *in vacuo* to about 400 ml.: sugar (80 g.) and NaH_2PO_4 (1 g.) in water (300 ml.) were added, the *p*H was adjusted to 6·0, and a further batch of yeast (40 g.) added. After a further 5 days' fermentation the yeast was filtered off,

the filtrate acidified and concentrated *in vacuo* to 20 ml. Excess of solid Na_2CO_3 was now added, and the mixture extracted with alcohol. The insoluble portion was worked up as described below. The alcoholic solution was treated with a little aqueous HCl and concentrated to low bulk. The aqueous residue was treated with excess Na_2CO_3 and repeatedly extracted with ether. The extracts were dried, concentrated to low bulk and treated with a solution of picric acid (1 g.) in ether; the picrate of 4-methyl-5-(β-hydroxyethyl)-thiazole was deposited as prisms M.P. 162°, 575 mg. or a yield of 57·5 % calculated on one only of the optical isomerides of the original amino-acid. (Picric acid found 61·2 %: $C_6H_9ONS.C_6H_3O_7N_3$ requires picric acid 61·6 %.) A mixed M.P. determination showed identity with the picrate of the synthetic compound.

From this picrate the picrolonate (M.P. 184°, decomp.) and *p*-nitrobenzoate (M.P. 125°) were obtained by the usual methods (yields 71 and 64 % respectively). In both cases mixed M.P. determinations showed identity with the corresponding derivatives of authentic 4-methyl-5-(β-hydroxyethyl)-thiazole. The *p*-nitrobenzoate gave C, 53·6; H, 4·14; S, 10·75 %. ($C_{13}H_{12}O_4N_2S$ requires C, 53·4; H, 4·11; S, 10·97 %.)

A blank experiment, identical with the above except that no amino-acid was added, gave none of the picrate of 4-methyl-5-(β-hydroxyethyl)-thiazole.

d(−)-α-*Amino-β-(4-methylthiazole-5)-propionic acid.* The alcohol-insoluble residue of the fermentation product (see above) was treated with water, and insoluble material filtered off. The filtrate was acidified to *p*H 1·5 with H_2SO_4 and phosphotungstic acid (20 % in water) added until no further immediate precipitation occurred. The precipitate was filtered off and washed once with a little water; the filtrate was treated with excess $Ba(OH)_2$, and after removal of barium phosphotungstate the Ba was quantitatively precipitated with H_2SO_4. The filtrate was concentrated *in vacuo* to low bulk, acidified to *p*H 7·0–7·4 with acetic acid, treated with an equal volume of alcohol and cooled. The amino-acid separated as colourless prisms (175 mg.): $[\alpha]_D = -9{\cdot}0°$ (1 % solution in *N* H_2SO_4). (Found: N, 15·01; S, 17·26; amino-N (Van Slyke) 7·35 %. $C_7H_{10}O_2NS$ requires N, 15·05; S, 17·2; amino-N, 7·5 %.) The compound gave a strongly positive ninhydrin reaction.

In an experiment in which the second fermentation was omitted the amino-acid isolated had $[\alpha]_D = -9{\cdot}5°$. The fact that the second fermentation does not appreciably alter the optical activity suggests that these specimens are optically pure.

α-*Amino-β-(4 methylthiazole-5)-propionic acid methyl ester dihydrochloride.* The amino-acid (II) (0·5 g.) was suspended in anhydrous methyl alcohol (20 ml.). HCl was passed in for 30 min. at room temperature and a further 30 min. with heating on the water bath. The solvent was removed *in vacuo* and the residue crystallized from methyl alcohol-ether. Yield 90 %; prisms, M.P. (decomp.) 187°. (Found: N, 10·15; Cl, 26·2 %. $C_8H_{14}O_2N_2SCl_2$ requires N, 10·25; Cl, 26·0 %.) In two experiments this compound was dissolved in chloroform containing diethylamine (1 equiv.), and benzyl chloroformate or acetyl chloride (1 equiv.) added. In neither case could a crystalline product be isolated.

The azlactone (*V*). 4-Methylthiazole-5-aldehyde [Harington & Moggridge, 1939] (1·0 g.), acetylglycine (0·92 g.), sodium acetate (3·2 g.) and acetic anhydride (9·4 ml.) were heated at 100° for 2 hr. On treatment with water the product separated in 25–30 % yield. Needles from acetic acid, M.P. 157·5°. (Found: N, 13·5 %. $C_7H_6O_2N_2S$ requires N, 13·48 %.)

α-*Acetamido-β-(4-methylthiazole-5)-acrylic acid methyl ester* (*VI*). The azlactone (V) was dissolved in methyl alcohol containing sodium methoxide (1 equiv.).

After 1 hr. at room temperature the mixture was acidified, the solvent removed *in vacuo*, and the residue recrystallized from water: needles M.P. 160°, yield 75–80%. (Found: N, 11·85%. $C_{10}H_{13}O_3N_2S$ requires N, 11·7%.)

α-Acetamido-β-(4-ethylthiazole-5)-propionic acid. The amino-acid (II) (1 g.), glacial acetic acid (10 ml.) and acetic anhydride (0·59 ml.) were boiled together for 1 min. The solvent was removed *in vacuo* and the residue crystallized from an ethyl acetate-petroleum mixture containing a little acetic acid. Needles M.P. 191°; 85% yield. (Found: N, 12·1%; equiv. by titration 226·5. $C_9H_{12}O_3N_2S$ requires N, 12·3%; equiv. 228.)

2-Methyl-4-amino-5-bromomethylpyrimidine hydrobromide (*IV*). This compound was prepared by the following modification of the method of Andersag & Westphal [1937]. 2-Methyl-4-amino-5-aminomethylpyrimidine dihydrochloride (2·11 g.) in water (8 ml.) was held at 60° with stirring while barium nitrite (1·237 g.) in water (10 ml.) was added gradually. The solution was kept at 60° for another hour, 2*N* H_2SO_4 (5·0 ml.) was added, the $BaSO_4$ filtered off and the filtrate concentrated *in vacuo*. The residual 2-methyl-4-amino-5-hydroxymethylpyrimidine hydrochloride was recrystallized from aqueous alcohol; yield 60%. This compound was heated with a 10% solution of HBr in acetic acid (40 vol.) at 100° for 2 hr. The precipitate was collected and recrystallized from methyl alcohol-ether. Yield 85–90%.

Attempts to condense the pyrimidine (*IV*) *with the amino-acid* (*II*). In various experiments the pyrimidine (IV) was mixed with equimolecular proportions of both the amino-acid (II) and the amino-acid monohydrobromide, and the mixtures heated (*a*) in water at 130°, and (*b*) without solvent at 150°. In no case was any evidence of reaction obtained; the amino-acid could be recovered as its picrate in yields in one case as high as 80%.

The N-acetamido-acid (*VII*). α-Acetamido-β-(4-methylthiazole-5)-propionic acid (0·794 g.) and 2-methyl-4-amino-5-bromomethylpyrimidine hydrobromide (1·0 g.) were ground together and heated without solvent to 160° for 30 min. with occasional stirring. The product was dissolved in methyl alcohol and carefully precipitated by addition of ether: yield 50%. It was recrystallized by the addition of acetone to the solution in methyl alcohol containing one drop of conc. aqueous HBr: small rod-like crystals, decomposing sharply at 260°. (Found: N, 13·4; Br, 31·1%. $C_{15}H_{21}O_3N_5SBr_2$ requires N, 13·7; Br, 31·3%.)

This compound (0·1 g.) was refluxed with *N* HBr (2·5 ml.) for 1 hr. the solvent removed *in vacuo* and the residue rubbed up with acetone: the precipitate was dissolved in water and treated with picric acid (0·148 g.) and 0·2*N* NaOH (3·2 ml.). The *tripicrate of the amino-acid* (*III*) was precipitated as an oil that hardened readily: M.P. (decomp.) 164°: yield 77·5%. (Found: picric acid 68·8%; $C_{13}H_{17}N_5O_2S.3C_6H_3O_7N_3$ requires picric acid 69·1%.) The amino-acid tribromide (III), as prepared either directly from the hydrolysis of the *N*-acetyl derivative (VII) or from the tripicrate, was usually obtained as small homogeneous but amorphous particles: in one experiment plates, M.P. (decomp.) 233°, were isolated, but in quantities too small for satisfactory analysis.

Summary

Yeast fermentation of *dl*-α-amino-β-(4-methylthiazole-5)-propionic acid leads to 4-methyl-5-(β-hydroxyethyl)-thiazole and the *d*(−)-form of the amino-acid.

The initial stages of a synthesis of the α-aminopropionic acid analogous to aneurin are described.

Our thanks are due to Prof. A. R. Todd for the gift of some synthetic 4-methyl-5-(β-hydroxyethyl)-thiazole: to Prof. Todd and to Messrs Hofmann-La Roche for gifts of 2-methyl-4-amino-5-aminoethylpyrimidine dihydrochloride: and to Messrs Inde Coope and Allsopp for the yeast used in these experiments.

REFERENCES

Andersag & Westphal (1937). *Ber. dtsch. chem. Ges.* **70**, 2035
Bonner & Buchman (1938). *Proc. Nat. Acad. Sci.* **24**, 431.
Harington & Moggridge (1939). *J. chem. Soc.* p. 443.

84. STUDIES IN DETOXICATION

7. THE BIOLOGICAL REDUCTION OF *l*-MENTHONE TO *d*-*NEO*MENTHOL AND OF *d*-*ISO*MENTHONE TO *d*-*ISO*MENTHOL IN THE RABBIT. THE CONJUGATION OF *d*-*NEO*MENTHOL WITH GLUCURONIC ACID

By R. TECWYN WILLIAMS

From the Physiology Department, Medical School, Hospitals Centre, Birmingham

(*Received 21 March 1940*)

A survey of the literature has shown that foreign ketones, when considered in relation to their fate in the mammalian organism, may be divided into two main groups.

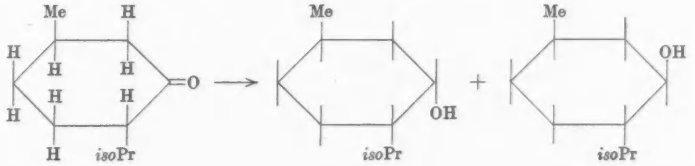
(1) *Aliphatic and aromatic ketones.* These undergo both oxidation and reduction in the body. As a result of biological reduction of the carbonyl group, secondary alcohols are produced which are usually excreted in conjugation with glucuronic acid. It should be noted that in these ketones the carbonyl group is the main point at which the organism attacks the molecule. Several aromatic ketones have been studied [cf. Thierfelder & Daiber, 1923] and acetophenone may be taken as a typical example. Besides being oxidized to benzoic acid [Nencki, 1878; Quick, 1928] and other products, a considerable proportion (36%) of it is reduced to methylphenylcarbinol [Thierfelder & Daiber, 1923]. Very few aliphatic ketones have been studied [cf. Neubauer, 1901], but Saneyoshi [1911] has shown that methylethylketone is reduced in rabbits, the reduction product, 2-butanol, being isolated from the urine as the glucuronide. *cyclo*-Hexanone is oxidized to adipic acid [Filippi, 1914; Bernhard, 1937].

(2) *Terpene ketones.* The ketones of this group that have been studied are all derivatives of bicyclic terpenes; very little work has been done on the monocyclic ketones. Unlike the ketones of the aliphatic and aromatic type, the carbonyl group of the bicyclic terpene ketones or camphors is very resistant to oxidation or reduction in the mammalian organism and usually survives unchanged, oxidation taking place elsewhere in the molecule with the production of a keto-alcohol which is usually excreted in conjugation with glucuronic acid. The following bicyclic ketones have been shown to behave in this manner, thujone [Hämäläinen, 1912; Hildebrandt, 1901], carone [Rimini, 1901], *d*-camphor [Asahina & Ishidate, 1933; 1934; 1935], *l*-camphor [Magnus-Levy, 1906], *r*-camphor [Mayer, 1908], *epi*camphor [Reinartz *et al.* 1934, 1], 5-oxocamphor [Reinartz *et al.* 1934, 2], dimethylcamphor [Reinartz & Meessen, 1939], *d*-fenchone [Reinartz & Zanke, 1936] and α-santenone (*π-nor*camphor) [Hämäläinen, 1912]. The only exception found so far is the camphane derivative, camphenilone, which was shown by Hämäläinen [1912] to undergo, in the rabbit, reduction of the keto group with the formation of camphenilol.

The fate of l*-menthone*

Three monocyclic terpene ketones have been studied without, however, any clear results. Hildebrandt [1902] showed that pulegone and carvone produced *in vivo* conjugated glucuronides of undetermined nature and Teppati [1937] claimed that pulegone was reduced to *d*-menthol and *l*-pulegol, although his evidence was unconvincing. Bonnani [1902] and Neubauer [1901] both demonstrated qualitatively that *l*-menthone gave rise, in rabbits, to a conjugated glucuronic acid. Later, Hämäläinen [1912] stated that *l*-menthone was oxidized in the rabbit to a tertiary keto-alcohol, probably 4-hydroxymenthone, but the evidence offered was inconclusive.

In the present paper definite evidence is presented to show that at least part of the *l*-menthone molecule undergoes reduction at the carbonyl group with the production of *d-neo*menthol which is excreted as a glucuronide. This glucuronide was isolated as the ammonium salt and, judging from the yield of this salt, at least 10–15 % of the *l*-menthone fed was reduced to *d-neo*menthol. Quantitative measurements of the glucuronic acid present in rabbit urine after *l*-menthone (see Table 1) showed that about 30–40 % of the menthone fed was converted into hydroxy derivatives which were excreted in conjugation with glucuronic acid. It has been shown by Read & Grubb [1934, 1] that reduction of *l*-menthone (I) with Ponndorf's reagent produced *d-neo*menthol (II) and *l*-menthol (III) in amounts of 70 and 30 % respectively. It is therefore possible that the reduction of



In the above formulae, the configurations used are those put forward by Read & Grubb [1934, 2].

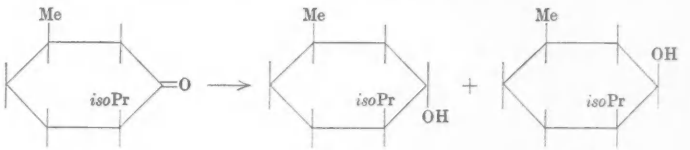
l-menthone *in vivo* would produce *l*-menthol in addition to *d-neo*menthol. *l*-Menthol, however, was not found, although its detection would have been a simple matter since its glucuronide has a high negative rotation ($[\alpha]_D - 110°$) compared with the other menthylglucuronides (see Williams [1939] for table of rotations). The possible explanations for the absence of *l*-menthylglucuronide other than experimental error, are (1) during the biological reduction of *l*-menthone, the production of the new optically active carbon atom is a completely asymmetric process, *d-neo*menthol only being formed, and (2) the biological reduction produces both menthanols (and here the process may be partially asymmetric or completely symmetric) but the rate of oxidation of *l*-menthol or its glucuronide is greater than that of *d-neo*menthol or its glucuronide and is such that no *l*-menthol survives at all. Some support is to be found for the second of these explanations, inasmuch as 67–68 % of *d-neo*menthol, when fed to a rabbit, is excreted in conjugation with glucuronic acid (see Table 3), whilst only 48 % of *l*-menthol is so conjugated [Williams, 1938]. It is not yet clear whether the oxidation and conjugation of menthols in the body are simultaneous or consecutive processes. Conjugation with glucuronic acid might well be an

intermediate stage in the biological oxidation of menthols. This point, however, is under investigation.

It appears, therefore, that *l*-menthone in its behaviour *in vivo* resembles the "aliphatic-aromatic" ketones since it undergoes reduction of its carbonyl group producing the secondary alcohol *d-neo*menthol. It may also resemble the camphors if Hämäläinen's [1912] claim, that it forms a keto-alcohol in which the original keto group still survives, can be substantiated.

The fate of d-iso*menthone*

Reduction of *d-iso*menthone (IV) should give *d-iso*menthol (V) and *d-neoiso*menthol (VI) [Read & Grubb, 1934, 3]. Hückel & Niggemeyer [1939] have shown that hydrogenation of *d-iso*menthone in acid solution in the presence of a Pt catalyst gave *d-neoiso*menthol, no *d-iso*menthol being formed. It would



IV V VI

be interesting to see what would happen to this ketone during biological reduction. A limited amount of *d-iso*menthone became available through the courtesy of Messrs Howard, Ltd., Ilford. Read & Grubb [1934, 3] state that *d-iso*menthone undergoes inversion to some extent into *l*-menthone; the material used in these experiments showed $[\alpha]_D + 78°$ compared with $+92°$ for the pure material, so that the ketone fed contained about 75% *d-iso*menthone, the rest being, presumably, *l*-menthone. On feeding 24 g. (dose 3 g./rabbit) of this ketone, 2·8 g. of crude ammonium menthanolglucuronate were isolated from the urine. The only substance definitely identified in this salt was *d-iso*menthylglucuronide which seemed to be the major if not the only substance present (this glucuronide has been previously described in Parts 2 and 4 of this series [Williams, 1938; 1940, 1]). It was hoped that the unknown *d-neoiso*menthylglucuronide would be isolated, but a thorough search for this compound could not be carried out owing to scarcity of *iso*menthone. It is hoped that the fate of this ketone can be reinvestigated at a later date when more material becomes available. However, on the basis of this single experiment it can be definitely stated that, like *l*-menthone, at least part of the *d-iso*menthone molecule is reduced in the rabbit to menthanols one of which is *d-iso*menthol.

The fate of d-neo*menthol*

It is shown in the present work that as far as that portion of *d-neo*menthol which undergoes conjugation with glucuronic acid is concerned, 67–68% of the material fed is conjugated. It has been shown in an earlier paper [Williams, 1938] that the *d*-menthanols, *d*-menthol and *d-iso*menthol, conjugate with glucuronic acid in the rabbit to the extent of 70 and 65% respectively, whereas only 48 and 45% respectively of the corresponding *l*-derivatives are so conjugated. The extent of conjugation of *d-neo*menthol is therefore of the same order as that of *d*-menthol and *d-iso*menthol. These three *d*-menthanols are structural isomerides (II, III, V) and it appears that this structural variation has no influence on their

conjugation with glucuronic acid in the body, although in purely chemical reactions such as the rate of formation of *p*-nitrobenzoates they show considerable differences [see Read & Grubb, 1934, 2]. Their *l*-antipodes conjugate to a much smaller extent with glucuronic acid [Williams, 1938].

Experimental

Experiments with l-*menthone*

Isolation of the conjugated glucuronide. Rabbits (2–3 kg.) on a diet of 100 g. cabbage and 50 g. bran a day were given 3 g. (3·3 ml.) of *l*-menthone[1] by stomach tube with water. The urine was collected during 2 days and precipitated with $(NH_4)_2SO_4$ (50 g./100 ml. urine) as described in earlier papers [e.g. Williams, 1940, 2]. The ammonium sulphate-treated urine was kept at 0° for 2 days and, during that time, a crystalline precipitate gradually settled, which proved to be crude *ammonium* d-neo*menthylglucuronate*. Various specimens of the crude dry salt showed $[\alpha]_D - 8$ to $-10°$ in water and the yield was 0·28–0·3 g./g. of *l*-menthone fed; a total of 14·4 g. of the salt was isolated after feeding 57 g. of the ketone. The urine after separation of the salt was made strongly acid with 20 % H_2SO_4 and thoroughly extracted with ether. The ethereal extract on evaporation left a clear brown syrup from which no crystalline material could be isolated. This syrup was mainly composed of glucuronides since it gave a very intense naphthoresorcinol reaction and smelt strongly of terpenes. Treatment of a portion of the syrup dissolved in alcohol with 2:4-dinitrophenylhydrazine gave a small amount of an unidentified hydrazone, but no *l*-menthone 2:4-dinitrophenylhydrazone was isolated. The syrup decolorized permanganate, but no definite evidence as to its composition has yet been obtained.

Isolation of d-neo*menthylglucuronide.* The above ammonium salt (4·5 g.) was dissolved in 200 ml. of water and the solution warmed and filtered. To the filtrate 15 ml. of 2*N* H_2SO_4 were added and a crystalline precipitate (3 g.) was immediately formed. A further quantity was obtained from the mother liquor by ether extraction. No *l*-menthylglucuronide was detected in the mother liquor. The above crystalline material after several recrystallizations from hot water was obtained pure and had M.P. 145° unchanged in mixture with authentic d-neo*menthylglucuronide* (see below). It formed long slender white needles, sparingly soluble in cold water but easily soluble in hot water, alcohol and ether; it showed $[\alpha]_D^{20°} - 15°$ ($c = 1·4$ in alcohol) and $[\alpha]_D - 10°$ ($c = 1$ in 0·8*N* NaOH). (Found: C, 54·9; H, 8·7; H_2O, 5·1 %; equiv. by titration, 349. $C_{16}H_{28}O_7$, H_2O requires C, 54·8; H, 8·6; H_2O, 5·1 %; equiv. 350.)

Hydrolysis of the glucuronide: isolation of d-neo*menthol.* The foregoing glucuronide (2 g.) was hydrolysed by steam-distilling with 20 ml. of 0·5*N* H_2SO_4. The *neo*menthol in the distillate was extracted with light petroleum and the extract dried with anhydrous Na_2SO_4. Evaporation of the petroleum left *d-neo*menthol as a colourless thick liquid (0·93 g.) with $n_D^{14°} = 1·4617$ and $[\alpha]_D^{22°} + 19·8°$ ($c = 2·3$ in alcohol); Read & Grubb [1934, 1] give $n_D^{17°} = 1·4617$ and $[\alpha]_D + 20·7°$ (in alcohol). It was further identified as the crystalline *p*-nitrobenzoate and 3:5-*dinitrobenzoate*. *d-neo*Menthyl *p*-nitrobenzoate from the above biosynthetic *d-neo*menthol showed $[\alpha]_D^{22°} + 18·96°$ ($c = 1$ in $CHCl_3$) and had M.P. 94° unchanged in mixture with an authentic specimen; Read & Grubb [1934, 1] give M.P. 94·5–95° and $[\alpha]_D + 17·8°$ (in $CHCl_3$). d-neo*Menthyl* 3:5-*dinitrobenzoate* prepared

[1] Howard, Ltd., Ilford. The menthone showed $[\alpha]_D^{14°} - 25·3°$ ($c = 2$ in alcohol) and was free from menthols, since no menthyl *p*-nitrobenzoates could be isolated from it on heating with *p*-nitrobenzoyl chloride in the presence of pyridine in the usual manner.

from the biosynthetic *neo*menthol formed long needles and after three recrystallizations from alcohol had M.P. 155° and showed $[\alpha]_D^{22°}+23{\cdot}6°$ ($c=0{\cdot}9$ in $CHCl_3$). (Found: C, 58·1; H, 6·1; N, 8·15%. $C_{17}H_{22}N_2O$ requires C, 58·25; H, 6·3; N, 8·0%.) Since this dinitrobenzoate has not been previously described an authentic specimen was prepared by condensing *d-neo*menthol (Eastman Kodak, Ltd.) with 3:5-dinitrobenzoyl chloride in the presence of pyridine in the usual manner. The authentic material formed long needles from alcohol, M.P. 155° (not depressed by admixture with the dinitrobenzoate from biosynthetic *d-neo*menthol) and $[\alpha]_D^{22°}+24{\cdot}8°$ ($c=0{\cdot}95$ in $CHCl_3$). Read & Grubb [1933] give M.P. 153° and $[\alpha]_D-23{\cdot}9°$ (in $CHCl_3$) for *l-neo*menthyl 3:5-dinitrobenzoate.

Quantitative determination of glucuronic acid in urine after feeding menthone. Rabbits on a standard diet were given *l*-menthone (dose 1 g./kg.) with water by stomach tube. The urine was collected for about 40 hr. and analysed for glucuronic acid by an adaptation of the Shaffer-Hartmann blood sugar method. For this purpose 1 ml. of the filtered urine was mixed with 1 ml. of 2% H_2SO_4 and extracted for 1 hr. with ether in a small (2 ml.) urine extractor. The ethereal extract was evaporated and the residue hydrolysed by boiling for 10 min. on a hot-plate with 5 ml. *N* HCl. The cooled solution was then exactly neutralized with *N* NaOH using a trace of methyl red as indicator (a drop from a capillary tube). The evaporation, hydrolysis and neutralization can be carried out in the boiling flask of the micro-extractor. The solution was now transferred quantitatively to a 25 ml.[1] measuring flask and made up to the mark. The glucuronic acid in this solution was determined on 5 ml. portions using the Shaffer-Hartmann reagent 50 of Shaffer & Somogyi [1933] (containing 150 ml. of KIO_3 solution equivalent to 0·1 *N* I_2). The glucurone content of the solution was then read off from a graph previously constructed from titrations of standard solutions of pure glucurone and sodium glucuronate. It was found that solutions of glucurone exactly neutralized in the cold with NaOH (i.e. sodium glucuronate) had the same reducing power as the equivalent of glucurone. The results for *l*-menthone are given in Table 1.

Table 1. *Glucuronic acid conjugation in the rabbit after* l-*menthone*

Rabbit no.	Wt. of rabbit, kg. and dose g.	Time of urine collection hr.	Glucurone found g.	Menthone conjugated g.	Menthone converted into hydroxy compounds %
61	2·0	43	0·9559	0·8254	41·3
62	2·4	43	0·8976	0·7752	32·3
63	2·6	43	0·8550	0·7384	28·4
61	2·0	48	0·9048	0·7918	39·6

Experiments with d-iso*menthone*

Isolation of an ammonium menthylglucuronate. Eight rabbits were each given 3 g. of *d-iso*menthone ($[\alpha]_D+78°$; $c=2$ in alcohol) with water by stomach tube. The urine was collected during 2 days and treated with $(NH_4)_2SO_4$ as previously described. After 2 days at 0° the urine contained a small amount of crystalline precipitate which was collected and dried (yield, 2·8 g.). The crude salt gave an intense naphthoresorcinol reaction and showed $[\alpha]_D-16{\cdot}3°$ ($c=1$ in water).

Isolation and identification of d-iso*menthylglucuronide.* The whole of the above salt was dissolved in 50 ml. water and the solution filtered and acidified with dilute H_2SO_4. On standing the solution deposited a nodular solid which was

[1] The dilution is so arranged that 5 ml. of the solution contain 0·5–1·5 mg. of glucurone.

coloured owing to adsorption of urinary pigments. The solid was recrystallized 4 times from hot water and eventually typical long white needles of *d-iso*menthylglucuronide were obtained, M.P. 124–125° and mixed M.P. 125–126° and showing $[\alpha]_D^{22°} -42{\cdot}9°$ ($c=1$ in alcohol) (Williams [1938] gives M.P. 126° and $[\alpha]_D -43{\cdot}2°$ in alcohol). It was further identified by isolation of *d-iso*menthol. A small amount of the glucuronide was hydrolysed by dilute acid and the *iso*menthol formed recovered by a simultaneous steam-distillation. The recovered *d-iso*menthol had M.P. 80° and mixed M.P. 80–81° (authentic *d-iso*menthol has M.P. 82–83°).

Experiments with d-neo*menthol*

Ammonium d-neo*menthylglucuronate*. Two rabbits were each given 3 g. (3·3 ml.) of *d-neo*menthol ($[\alpha]_D^{22°} +22°$, $c=2$ in alcohol) (Eastman Kodak, Ltd.) with water by stomach tube. The urine was collected during 2 days and crude ammonium *d-neo*menthylglucuronate (5·2 g.) isolated from it by the ammonium sulphate method [cf. Williams, 1940, 2]. The crude salt showed $[\alpha]_D^{20°} -5{\cdot}2°$ ($c=2$ in water). It was dissolved in the minimum of water with warming and the solution was filtered. Acetone (2–3 vol.) was then added whereby a small precipitate was formed. This was discarded since it was mainly $(NH_4)_2SO_4$ and acetone was now added to the solution in excess thereby precipitating *ammonium* d-neo*menthylglucuronate* in an apparently amorphous state. On standing, the salt crystallized and was then recrystallized from aqueous acetone. The salt formed long matted needles and after drying *in vacuo* over $CaCl_2$ contained 1 mol. H_2O. The monohydrate showed $[\alpha]_D -5{\cdot}9°$ ($c=2$ in water) and the anhydrous salt (dried at 110°) showed $[\alpha]_D^{20°} -6{\cdot}9°$ ($c=2$ in water). (Found: glucurone by Shaffer-Hartman, 47·7; H_2O, 4·6 %. $C_{16}H_{27}O_7NH_4$, H_2O requires glucurone, 47·9; H_2O, 4·9 %.)

Preparation of d-neo*menthylglucuronide*. (*a*) Rabbits were given *d-neo*menthol as described above. After 2 days the urine was collected, filtered through muslin and treated with 1/20 vol. of 20 % H_2SO_4. It was kept at 0° for about an hour and during that time *d-neo*menthylglucuronide separated as long needles. The crystals were filtered off under suction and dried in a desiccator. The yields of the crude acid are given in Table 2; only about 50 % of the acid in the urine could be recovered in this manner and the greater the volume of fluid the less was the yield.

Table 2. *Preparation of* d-neo*menthylglucuronide*

Rabbit no.	Dose g.	Time of urine collection hr.	Vol. of urine + washings ml.	Yield of crude acid g.
59, 60	3	48	600	3·2
67, 70	3	48	510	3·8
68, 69	3	48	480	4·1
56	2·4	24	164	1·5

(*b*) An aqueous solution of the ammonium salt was acidified with dilute H_2SO_4 and *d-neo*menthylglucuronide immediately separated as fine felted needles. After two recrystallizations from water, the acid had M.P. 146° and showed $[\alpha]_D^{22°} -14{\cdot}6°$ ($c=2$ in alcohol).

Quantitative determination of glucuronic acid in urine after d-neo*menthol*. Rabbits were given *d-neo*menthol (dose 1 g./kg.) with water by stomach tube.

The urine collected was analysed for glucuronic acid by the Shaffer-Hartmann method as described above in the section on *l*-menthone. The results are given in Table 3.

Table 3. *The conjugation of* d-neo*menthol with glucuronic acid in the rabbit*

Rabbit no.	Wt. of rabbit, kg. and dose g.	Time of urine collection hr.	Glucurone found g.	*neo*Menthol conjugated g.	% *neo*Menthol conjugated
56	2·38	24	1·427	1·248	52·4
61	2·02	43	1·582	1·384	68·5
64	2·23	43	1·714	1·499	67·2
66	2·50	43	1·915	1·675	67·0

Summary

The fate of *l*-menthone, *d-iso*menthone and *d-neo*menthol in the rabbit has been studied.

It has been found that on administration of *l*-menthone to rabbits, about 30–40 % of it is excreted as hydroxy derivatives conjugated with glucuronic acid. Isolation of *d-neo*menthylglucuronide from the urine indicates that at least part of the menthone molecule is reduced at the carbonyl group. About 10–15 % of the *l*-menthone fed was actually isolated as *d-neo*menthylglucuronide.

*d-iso*Menthone is also reduced in the rabbit, *d-iso*menthol (isolated as the glucuronide) being identified as a reduction product.

On feeding *d-neo*menthol to rabbits, 67–68 % was excreted in the urine combined with glucuronic acid; this figure is of the same order as those previously found for *d*-menthol (70 %) and *d-iso*menthol (65 %).

A method is described, using a Shaffer-Hartmann reagent, for the quantitative estimation of conjugated glucuronic acid in 1 ml. urine after feeding menthol derivatives.

The following compounds are described for the first time: *d-neo*menthylglucuronide and its ammonium salt and *d-neo*menthyl 3:5-dinitrobenzoate.

References

Asahina & Ishidate (1933). *Ber. dtsch. chem. Ges.* **66**, 1673.
—— —— (1934). *Ber. dtsch. chem. Ges.* **67**, 71.
—— —— (1935). *Ber. dtsch. chem. Ges.* **68**, 947.
Bernhard (1937). *Hoppe-Seyl. Z.* **248**, 256.
Bonnani (1902). *Arch. Farmacol. sper.* **1**, 469, 498.
Filippi (1914). *Arch. Farmacol. sper.* **18**, 178 (cited by Bernhard, 1937).
Hämäläinen (1912). *Skand. Arch. Physiol.* **27**, 141.
Hildebrandt (1901). *Arch. exp. Path. Pharmak.* **45**, 110.
—— (1902). *Hoppe-Seyl. Z.* **34**, 456.
Hückel & Niggemeyer (1939). *Ber. dtsch. chem. Ges.* **72**, 1354.
Magnus-Levy (1906). *Biochem. Z.* **2**, 319.
Mayer (1908). *Biochem. Z.* **9**, 439.
Nencki (1878). *J. prakt. Chem.* **18**, 288.
Neubauer (1901). *Arch. exp. Path. Pharmak.* **46**, 152.
Quick (1928). *J. biol. Chem.* **80**, 515.

Read & Grubb (1933). *J. chem. Soc.* p. 167.
—— —— (1934, 1). *J. Soc. chem. Ind., Lond.*, **53**, 52 T.
—— —— (1934, 2). *J. chem. Soc.* p. 1779.
—— —— (1934, 3). *J. chem. Soc.* p. 316.
Reinartz & Meessen (1939). *Ber. dtsch. chem. Ges.* **72**, 1.
—— & Zanke (1936). *Ber. dtsch. chem. Ges.* **69**, 2259.
—— —— & Faust (1934, 1). *Ber. dtsch. chem. Ges.* **67**, 589.
—— —— —— & Schaefers (1934, 2). *Ber. dtsch. chem. Ges.* **67**, 1536.
Rimini (1901). *Chem. Z.* **1**, 1227 (cited by Hämäläinen, 1912).
Saneyoshi (1911). *Biochem. Z.* **36**, 22.
Shaffer & Somogyi (1933). *J. biol. Chem.* **100**, 695.
Teppati (1937). *Arch. int. Pharmacodyn.* **57**, 440.
Thierfelder & Daiber (1923). *Hoppe-Seyl. Z.* **130**, 380.
Williams (1938). *Biochem. J.* **32**, 1849.
—— (1939). *Biochem. J.* **33**, 1519.
—— (1940, 1). *Biochem. J.* **34**, 48.
—— (1940, 2). *Biochem. J.* **34**, 272.

85. THE EXCRETION OF AN ANTI-PERNICIOUS ANAEMIA PRINCIPLE IN THE URINE

By JOHN FREDERICK WILKINSON, LOUIS KLEIN, CHARLES AMOS ASHFORD, TUDOR STANLEY GEORGE JONES, BRIAN RHODERICK SUTTON MAINWARING and FRANCIS XAVIER AYLWARD

From the Department of Clinical Investigation and Research, Royal Infirmary and University of Manchester

(*Received 27 March 1940*)

Research on pernicious and other megalocytic anaemias has indicated that in normal gastro-haemopoiesis [Wilkinson, 1936] at least 3 unidentified chemical factors are involved. These are an "intrinsic" factor [Castle, 1929, 1] or haemopoietin [Wilkinson & Klein, 1932; Klein & Wilkinson, 1933; 1934] which has enzyme-like properties, and is present in the secretion and mucous membrane of the stomach and in the pylorus, and apparently reacts with an "extrinsic factor" present in foodstuffs such as beef protein [Castle, 1929, 2; Castle & Townsend, 1929; Castle *et al.* 1930; Jones *et al.* 1938], producing a third factor identical with or similar to the anti-pernicious anaemia principle present in liver [Klein & Wilkinson, 1934]. The latter principle is absorbed from the intestine and stored chiefly in the liver, but may be present in other tissues such as the kidney [McCann, 1928], lungs [Hitzenberger, 1934], brain [Ungley, 1931] and salivary gland and pancreas [Dakin *et al.* 1936].

The liver, however, appears to be the main depot and the active principle is transported in the blood to the bone marrow when required for normal erythropoiesis [Wilkinson & Klein, 1934; Wilkinson *et al.* 1937].

Under certain circumstances it would appear that this liver factor might be excreted in the urine. This has not been clearly established, although it is a matter not only of theoretical but also practical importance, for example in furnishing a possible means for the early diagnosis of pernicious anaemia. Some attempts have already been made to detect the liver factor in urine but they have only provided inconclusive evidence owing to the conflicting results obtained and the unsatisfactory nature of the tests employed. This may be due partly to the difficulty of separating without loss of potency small quantities of the anti-pernicious anaemia principle from large quantities of urine in a state suitable for parenteral injection, free from toxic substances, and partly to the fact that many of the examinations for anti-pernicious anaemia potency have been carried out on rats, guinea-pigs or pigeons which have been shown repeatedly by many reliable observers to be quite unsuitable for such tests, since a non-specific reticulocytosis (*a*) may arise spontaneously, by change of diet, or under the influence of substances present in liver other than the active anti-pernicious anaemia principle, or (*b*) may even fail to occur following the administration of a known active liver extract. In such a manner did Liener [1935], Walters [1936], Jéquier & Apsley [1938] test their extracts prepared from the urines of patients with pernicious anaemia collected before and during liver treatment and their results therefore are open to criticism.

The only reliable method of assay of the anti-pernicious anaemia factors so far is the clinical test applied under carefully controlled conditions [cf. Wilkinson, 1932]. Unfortunately, the very few such tests that have been carried out on urine extracts have given contradictory results.

Wakerlin [1935] and Wakerlin *et al.* [1935], after preliminary experiments on pigeons, obtained a positive clinical response in the treatment of one case of pernicious anaemia with a concentrated extract of normal urine, while Decastello [1935] obtained similar positive results. In later experiments, however, Wakerlin & Brunner [1936], and Wakerlin [1937; 1940] were unable to confirm their previous work, while Thornley [1938] obtained a negative result with an extract of normal urine tested on one case of pernicious anaemia. Wilkinson [1938] reported some preliminary results obtained in his clinic showing that haemopoietically active extracts could be obtained from normal urine and from urine collected from treated and remitting cases of pernicious anaemia with subacute combined degeneration of the spinal cord. In this paper we present further evidence regarding the presence of an haemopoietically active anti-anaemic factor in normal and certain other urines.

Experimental

Extracts were prepared from fresh urines by two methods, either by direct concentration *in vacuo* and subsequent extraction or, alternatively, by an adsorption technique. Three of these were made from normal urines, two from the urines of untreated patients suffering from pernicious anaemia in relapse, and four from the urines of treated patients with remitted pernicious anaemia, with or without symptoms and signs of subacute combined degeneration of the spinal cord. 20–40 l. of urine were used in each case and details of the extractions are shown in Table 1.

Table 1. *Extracts of human urines*

Extract no.	Urine	Method of extraction	Volume of urine extracted l.	Wt. of dry extract g.	Haemopoietic and clinical activity
U 3	Normal male	Direct concentration	40·0	32·9	Discarded
U 4		Norite adsorption	40·0	11·7	Active
U 9		Direct concentration	20·0	18·0	Active
U 1	Treated remitting pernicious anaemia	Direct concentration	46·5	41·0	Active
U 2		Direct concentration	49·0	100·0	Active
U 6		Norite adsorption	23·0	8·5	Discarded
U 8		Direct concentration	20·0	54·0	Active
U 5	Untreated relapsing pernicious anaemia	Norite adsorption	35·7	10·9	Inactive
U 7		Direct extraction	20·0	50·0	Inactive

(i) *Direct concentration method*

The samples of fresh urine were concentrated daily as soon as obtained to about one-tenth of their volumes at a temperature below 30° *in vacuo* using a Reich distillation apparatus. An equal volume of 90% alcohol was then added; the corresponding daily samples of the concentrates were mixed and later filtered. The filtrate was concentrated to one-tenth of its volume and two volumes of 90% alcohol were added. The resulting precipitate was filtered off and the filtrate after concentration to a syrup was poured into a large excess of absolute alcohol. The precipitate obtained was extracted with warm absolute alcohol to remove urea and other impurities and was then washed with ether, dried in a vacuum desiccator and weighed.

(ii) *Norite adsorption method*

The urine was treated in batches of 2 l. with 20 g. of norite at *p*H 5 and after standing for 24 hr. the norite was removed by filtration and put into 400 ml. of 60 % alcohol. The daily samples, left at this stage until all had been collected, were then mixed and boiled for 30 min. After filtration the norite was extracted twice more with 1 l. of phenol, containing about 5 % water and the extract was treated with ether and water. The aqueous layer was mixed with the 60 % alcohol filtrate and concentrated *in vacuo* to a syrup, which was poured into an excess of absolute alcohol. The resulting precipitate was washed with absolute alcohol, then ether and dried in a desiccator.

The clinical assay of the urinary extracts

The products were prepared for intramuscular administration by solution in the minimal amounts of distilled water, centrifuging and sterilization by passage through a Seitz filter.

The potency of the extracts was determined by the haematological and clinical effects following their intramuscular injection, after preliminary control periods, into well authenticated and specially controlled patients with relapsing pernicious anaemia. Haemopoietically active extracts produced firstly a sharp rise or peak in the number of reticulocytes in the circulating blood, and this was followed by an increase in the red blood cells and in the percentage of haemoglobin, with a simultaneous improvement in the clinical condition. The patients used for this purpose were all carefully chosen according to the rigid criteria laid down by one of us [Wilkinson, 1932]; estimations of the reticulocytes were made daily, and of the red cells, white cells and haemoglobin one or twice weekly as indicated by the state of the patient.

Results

There were three groups of experiments.

(i) *Extracts from normal urines*

Extracts U 3, U 4 and U 9 were prepared from the urines collected from normal individuals in the Department, none of whom was taking liver in any form.

Extract U 3 gave painful reactions and was discarded, but the other two, U 4 and U 9, that were prepared differently, gave good positive clinical responses when given intramuscularly to test patients in doses equivalent to 8·5–20 l. of urine.

Case PA/833, with an initial count of, red cells 1,570,000 per μl., haemoglobin 42 %, reticulocytes 0·1 %, received on 17 December 1937 2·5 g. of extract U 4 in one dose intramuscularly (equivalent to 8·5 l. of urine) and showed 14·8 % reticulocytes on the 9th day, and on the 17th day, red cells 2,340,000 per μl., haemoglobin 55 %, with a corresponding good clinical improvement.

Case PA/851, with an initial count of, red cells 1,420,000 per μl., haemoglobin 34 % and reticulocytes 0·2 %, received on 20 January 1938 2·4 g. of extract U 4 in 4 equally divided doses (equivalent to 8·2 l. of urine) followed by 0·6 g. on each of the 9th, 10th, 13th and 14th days later (total 4·8 g. extract equivalent to 16·4 l. of urine); unfortunately the maximal reticulocyte peak was accidentally missed but the reticulocytes were 11·6 % on the 12th day and on the 19th day the count was, red cells 2,040,000 per μl., haemoglobin 52 %

LINDA HALL LIBRARY
KANSAS CITY, MO.

and reticulocytes 1·4%, with marked clinical improvement. Similarly, case PA/ADD/146, with an initial count on 6 October 1939 of, red cells 1,400,000 per μl. and haemoglobin 28%, received 18 g. of extract U 9 (equivalent to 18 l. of urine) and showed a reticulocyte response of 12·5% on the 11th day and subsequent improvement in the blood and clinical condition.

(ii) *Urine extracts from treated patients with pernicious anaemia in remission*

These patients were in-patients under our control and had been given the usual anti-anaemic treatment; two of them had marked symptoms of subacute combined degeneration of the spinal cord. All had responded to anti-anaemic treatment and had red cell counts greater than 4,000,000 per μl. at the times when the urines were collected.

Extracts U 1, U 2 and U 8 were prepared by the direct concentration method and all gave positive clinical responses when given intramuscularly to test patients in total dosages equivalent to 10–22·6 l. of urine. Extract U 6 gave toxic reactions and was discarded.

Case PA/627, with an initial count on 12 February 1936 of, red cells 1,280,000, haemoglobin 40%, reticulocytes 1·0%, received 20 g. extract U 1 in 8 days (equivalent to 22·6 l. urine); the reticulocytes rose to 12·2% and on the 24th day the count was, red cells 2,100,000 per μl., haemoglobin 63% and reticulocytes 5·8%, with very marked clinical improvement.

Case PA/633, with an initial count on 25 February 1936 of, red cells 1,420,000 per μl., haemoglobin 38% and reticulocytes 0·9%, received 34·5 g. extract U 2 over 11 days (equivalent to 15 l. of urine); the reticulocytes rose to 29·6% on the 15th day, and on the 21st day the red cells were 2,510,000 per μl., haemoglobin 62% and reticulocytes 6·9% (on the 28th day, red cells 2,760,000 per μl., haemoglobin 74%), with considerable clinical improvement.

Case PA/975, with an initial count on 27 June 1939 of, red cells 1,730,000 per μl., haemoglobin 43% and reticulocytes 2·4%, received 29 g. extract U 8 over 11 days (equivalent to 10·7 l. of urine); the reticulocytes rose to 15·2%, and on the 21st day the red cells were 2,200,000 per μl., haemoglobin 54%; on the 25th day, the red cells were 3,200,000 per μl. and haemoglobin 74%, with a progressive clinical improvement.

(iii) *Urinary extracts from untreated patients with relapsing pernicious anaemia*

These in-patients were suffering from severe relapsing pernicious anaemia and did not receive any anti-anaemic treatment until about 40 l. of urine had been collected from each of them. Extracts U 5 and U 7 were prepared by absorption on norite and direct concentration respectively, both failing to produce any haemopoietic responses when injected intramuscularly into three test patients. Thus, case PA/853 (initial count on 15 March 1938 of, red cells 1,150,000 per μl., haemoglobin 31%, reticulocytes 0·2%) received 2·5 g. extract U 5 on each of 3 successive days (a total of 7·5 g. equivalent to 24·7 l. of urine) without any response, the red cells falling to 800,000 per μl., and the haemoglobin to 21%; the subsequent response to an active liver extract was perfectly satisfactory.

Similarly, case PA/969, with an initial count on 24 June 1939 of, red cells 1,770,000 per μl., haemoglobin 48% and reticulocytes 0·8%, and case PA/971, with an initial count on 4 July 1939 of, red cells 1,660,000 per μl., haemoglobin 42% and reticulocytes 0·8%, failed to respond to intramuscular injections of extract U 7, but showed normal and prompt remissions when subsequently given ordinary active anti-anaemic preparation.

Discussion

Our results show that from large quantities of normal urine it is possible to prepare extracts which, when administered to untreated patients suffering from relapsing pernicious anaemia, will initiate normal remissions in the same way that similarly prepared extracts of beef liver will do. While the urines from relapsing cases of pernicious anaemia failed to furnish such active extracts, when these patients had been adequately treated and their bloods and general conditions returned to normal they then passed urine from which active extracts could again be prepared.

There is, as yet, no evidence to show whether the anti-anaemic activity of these urinary extracts is due to a precursor of or to the actual so-called anti-pernicious anaemia liver principle, or to some other allied substance with similar haemopoietic properties; on theoretical grounds we would expect that the blood of normal individuals would contain the liver anti-anaemic principle in process of transport from the liver to the bone marrow. It is, therefore, reasonable to suppose that the principle present in the urine (admittedly in small quantities) is identical with this liver principle and represents that which has been removed via the kidneys from the blood. In relapsing pernicious anaemia, in which the liver stores are empty, there is none in the blood to be lost; on the other hand, in individuals receiving active anti-anaemia therapy the liver stores and presumably the blood level are restored [Wilkinson & Klein, 1934; Wilkinson *et al.* 1937] and the principle therefore appears once more in the urine.

In view of the difficulties, on the one hand, associated with attempts to express the activity of anti-anaemia extracts in a quantitative form on the basis of results of clinical tests on relatively few cases, and, on the other hand, encountered in preparing extracts readily from large amounts of urine, it is obvious that the examination of the urine for anti-pernicious anaemia potency does not offer a suitable or convenient diagnostic test for pernicious anaemia. If, however, an accurate chemical or biological test becomes available by which relatively small amounts of urine can be accurately assayed then such a test may be of great value in the early diagnosis of the disease, since absence of the anti-anaemia factor from the urine should be one of the first signs long before the blood picture becomes characteristic.

These considerations raise the possibility of a renal threshold for the anti-pernicious anaemia factor; the occurrence of a low renal threshold for the liver principle could under certain circumstances result in a continuous excretion of the liver factor and ultimately a deficiency sufficient to cause a megalocytic anaemia—this may indeed be the explanation of the occurrence of such types of anaemia in association with certain renal conditions.

Summary

1. Extracts have been prepared from normal male urines and from the urines of treated patients with remitting pernicious anaemia which will initiate normal remissions of the disease when given intramuscularly to relapsing patients with pernicious anaemia.

2. Similar extracts prepared from the urines of untreated relapsing cases of pernicious anaemia did not possess such anti-pernicious anaemia activity when tested clinically.

3. This urinary anti-pernicious anaemic principle although showing a similar clinical action may or may not be identical with the liver principle.

4. This active material is present in the urine in relatively small amounts.

This work was carried out with the assistance of Research Grants from the Medical Research Council.

REFERENCES

Castle (1929, 1). *Proc. roy. Soc. Med.* **22**, 1214.
—— (1929, 2). *Amer. J. med. Sci.* **178**, 748.
—— & Townsend (1929). *Amer. J. med. Sci.* **178**, 764.
—— —— & Heath (1930). *Amer. J. med. Sci.* **180**, 305.
Dakin, Ungley & West (1936). *J. biol. Chem.* **115**, 771.
Decastello (1935). *Med. Klinik.* **31**, 377.
Hitzenberger (1934). *Klin. Wschr.* **13**, 1345.
Jéquier & Apsley (1938). *Brit. med. J.* **2**, 934.
Jones, Grieve & Wilkinson (1938). *Biochem. J.* **32**, 665.
Klein & Wilkinson (1933). *Biochem. J.* **27**, 600.
—— —— (1934). *Biochem. J.* **28**, 1684.
Liener (1935). *Wien. klin. Wschr.* **48**, 559.
McCann (1928). *Proc. Soc. exp. Biol., N.Y.*, **25**, 255.
Thornley (1938). *Brit. med. J.* **2**, 1113.
Ungley (1931). *Lancet*, **2**, 63.
Wakerlin (1935). *Proc. Soc. exp. Biol., N.Y.*, **32**, 1607.
—— (1937). *Amer. J. Physiol.* **119**, 417.
—— (1940). *Arch. intern. Med.* **65**, 21.
—— & Brunner (1936). *Arch. intern. Med.* **57**, 1032.
—— —— & Kinsman (1935). *Amer. J. Physiol.* **112**, Proc. 136.
Walters (1936). *Amer. J. Physiol.* **116**, 158.
Wilkinson (1932). *Brit. med. J.* **1**, 325.
—— (1936). Brit. Encylopaedia of Medicine, **1**, 408. London: Butterworth and Co. Ltd.
—— (1938). *Brit. med. J.* **2**, 1110.
—— & Klein (1932). *Lancet*, **1**, 719.
—— —— (1934). *Quart. J. Med.* **3**, 341.
—— —— & Ashford (1937). *Quart. J. Med.* **6**, 143.

86. FAT METABOLISM IN FISHES

14. THE UTILIZATION OF THE ETHYL ESTERS OF FATTY ACIDS BY THE EEL AND THEIR EFFECT ON DEPOT FAT COMPOSITION

By JOHN ARNOLD LOVERN

From the Torry Research Station, Aberdeen, of the Department of Scientific and Industrial Research

(*Received 1 April 1940*)

In previous papers in this series [e.g. Lovern, 1937] the theory has been advanced that certain fish have the ability to hydrogenate or dehydrogenate their depot fats according to their specific requirements in relation to the type of fat ingested. Similar metabolic processes have been suggested for various mammals [Banks & Hilditch, 1931; 1932; Hilditch, 1937; Schoenheimer & Rittenberg, 1936; Rittenberg & Schoenheimer, 1937; Longenecker, 1939]. The theory has yet to be verified, however, by feeding an individual fatty acid at a high level and observing an increase in the corresponding saturated or unsaturated acid.

Using eels as experimental animals, an attempt has been made to obtain such evidence by feeding certain ethyl esters. The acids chosen were myristic, palmitic and the mixed unsaturated acids of eel fat. Ingestion of either of the first two acids in quantity should raise the content of saturated acids in the eel's depot fat. If the theory is correct and applies to the eel, this should lead, in turn, to a compensatory dehydrogenation of some of the saturated acid, and since tetradecenoic acid is not normally an important constituent of fish fats, the probability would be that palmitic acid would be dehydrogenated. Thus feeding of either ethyl myristate or palmitate might be expected to raise the content of hexadecenoic acid in the eel's depot fat. Conversely, feeding of unsaturated esters should lead to a compensatory hydrogenation process, and since there would be little tetradecenoic acid available for this and as stearic acid (and higher saturated acids) are not usually present in other than very small quantities in fish fats, the expectation would be that hydrogenation of hexadecenoic acid would be the most important process. Such changes, if of sufficient magnitude, should be detectable by comparing the fatty acid compositions of the fats from the experimental eels and normal eels.

Experimental

In the first experiments batches of 35 eels of 30–35 cm. in length were confined in large tanks supplied with slowly-running water. No temperature control was incorporated as the experiment was made during the summer when feeding should be satisfactory at the prevailing temperature. Attempts to feed the esters directly by pipette were unsatisfactory owing to subsequent vomiting. Accordingly the esters were injected subcutaneously into earthworms (killed by freezing) and such prepared worms were readily swallowed by the eels. If too large a quantity of an ester was injected into a worm at the start of the experiment, such a worm led to subsequent vomiting, but by gradually increasing the

amount the eels in the course of about a week were able to take as much ester as the worms could contain (up to 1 ml. per worm), without apparent digestive disturbances. The number of worms eaten per tank per day was noted, from which an approximate idea of the ester consumption was obtained. The ethyl myristate and palmitate were mixed with 15 and 20% respectively of eel fat, to furnish on digestion a mixture of acids rather than a single acid, since the work of Cox [1933] suggests that better absorption is thereby possible.

Ethyl palmitate and the unsaturated esters were readily acceptable to the eels (treated worms being taken almost as readily as untreated ones), but ethyl myristate was not well received and consumption throughout the experiment was very poor and erratic.

After a few weeks of fairly satisfactory feeding, the daily consumption began to decline, possibly owing to falling water temperatures. Feeding was continued, however, until all the prepared esters had been given when the fish were killed and the body fats extracted and analysed. The results are given in Tables 1 and 2.

Table 1. *Details of feeding*

Tank no. ...	1 (Myristate)	2 (Palmitate)	3 (Unsatd. esters)
1st Series (35 eels per tank)			
Initial wt. eels (g.)	3247	2757	2984
Final wt. eels (g.)	2411	2019	2326
	Loss 836	Loss 738	Loss 658
Duration of experiment (weeks)	14	14	14
Esters eaten (ml.)	95	178	234
Final fat content of eels (%)	17·4	13·1	14·7
2nd Series (35 eels per tank)			
Initial wt. eels (g.)	—	1952	1959
Final wt. eels (g.)	—	1958	1814
		Gain 6	Loss 145
Duration of experiment (weeks)	—	11	8·5
Esters eaten (ml.)	—	312	243
Final fat content of eels (%)	—	8·8	8·9

Table 2. *Composition of fatty acids of eel body fats* (%)

	Saturated			Unsaturated				
Material	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Untreated eels	4·3	17·3	2·1	0·1	9·0 (−2·2 H)	28·9 (−2·6 H)	20·4 (−5·8 H)	7·9 (−9·7 H)
Fed myristate	5·5	17·3	2·2	1·3	7·6 (−2·2 H)	35·8 (−2·7 H)	18·9 (−5·8 H)	11·4 (−10·0 H)
Fed palmitate (1)	5·5	18·7	1·5	0·7	8·8 (−2·1 H)	36·9 (−2·6 H)	17·9 (−5·7 H)	10·0 (−9·2 H)
Fed palmitate (2)	5·8	25·7	2·2	0·4	9·1 (−2·2 H)	37·1 (−2·5 H)	13·6 (−5·6 H)	6·1 (−8·5 H)
Fed unsaturated esters (1)	5·4	17·8	1·4	0·7	7·1 (−2·1 H)	39·3 (−2·8 H)	18·2 (−6·1 H)	10·1 (−9·3 H)
Fed unsaturated esters (2)	5·4	16·0	2·1	—	6·0 (−2·2 H)	42·6 (−2·6 H)	20·9 (−5·2 H)	7·0 (−10·5 H)

In view of the losses in weight during the experiment and the failure appreciably to modify the fatty acid composition, a second series of experiments was undertaken the following summer, using an elevated and controlled water temperature (20°), which has already been shown to increase feeding intensity

[Lovern, 1938]. This led to a much more regular and higher food consumption, but once again ethyl myristate proved unacceptable and its feeding was discontinued. The results of these later experiments are also given in Tables 1 and 2. The figures for ester consumption have been corrected for admixed eel fat. The worms themselves contained less than 2% of fat.

Discussion

The loss in weight of all the eels in the first experiments is in keeping with the results of earlier work [Lovern, 1938] where it was found that captive eels only gained in weight when the calorie intake was considerable. A curious feature of these eels is the high content of fat (%) at the end of the experiment. From their length they should have started with about 9–10% of fat [Lovern, 1938], and, in fact, a sample tested gave such a figure. These eels were also unusually thick for their size, and this is reflected in the higher weights of 35 fish in exp. 1 than in exp. 2, where the eels were also roughly of the same length. From all the feeding experiments on eels which the writer has carried out, it would appear that these fish do not necessarily utilize primarily fat during partial starvation, but may use protein instead, thus actually having a higher fat percentage after loss of weight. (This seems to apply especially in cold water.)

In the second experiments there was no great change in total weight and the fat content was normal for eels of that size.

From Table 2 it can be seen that whilst in exp. 1 the results have been in general what would be expected if the theory of interchangeability is correct, the extent of the changes is, in general, too small to be outside experimental error. On the myristate diet the most notable effect has been an increase in tetradecenoic acid, which, although small, is probably outside experimental error. The composition given for untreated eels is an average of two analyses for eels of 30–35 and 50–55 cm. long respectively [see Lovern, 1938]. These analyses agreed closely and from all other eel fat results given in this earlier paper it would seem that a figure of 0·1% of tetradecenoic acid is not likely to be greatly exceeded. The contents of myristic and tetradecenoic (with one exception) acids are apparently higher than normal in all the fats from eels fed on earthworms and this may be due to ingestion and deposition of small amounts of lauric and dodecenoic acids derived from the worm fat [see following paper]. Traces of such acids would not be detectable as such in the eel fats, but would result in apparently higher contents of C_{14} acids at the expense of the C_{16} acids. The only previous eel fats encountered with more than about 4·5% of myristic acid were from fish fed on herring, the fat of which contained a relatively high content of myristic acid [Lovern, 1938]. If the abnormally high content of tetradecenoic acid in the fat from eels fed with myristate has any significance, it suggests a desaturation process during absorption in this case, rather than after deposition, since the stimulus of an abnormally high content of saturated acids in the depot fat has not been effected.

On the palmitate diet in exp. 1 there has been a slight rise in the palmitic acid figure, but no corresponding rise in hexadecenoic acid. The fat from the eels fed on unsaturated esters has a rather low content of hexadecenoic acid, whereas it should have tended to rise slightly if no hydrogenation had occurred. The unsaturated esters fed had the following percentage fatty acid composition: sat. acids, C_{14}, 2·4; C_{16}, 5·7: unsat. acids, C_{14}, 0·5; C_{16}, 11·5 (−2·3 H); C_{18}, 45·4 (−2·8 H); C_{20}, 25·8 (−5·9 H); C_{22}, 8·7 (−9·2 H). However, once again the stimulus of an abnormally low content of saturated acids in the depot fat is

lacking, and if hydrogenation of hexadecenoic acid has occurred it has probably been during the absorption processes.

Turning to the second series of experiments, the results have been in the same direction, but more definite. On the palmitate diet the content of palmitic acid has risen by 8·4 units, a figure far beyond experimental error and undoubtedly due to assimilation of palmitic acid from the ethyl palmitate. It is noteworthy that this should be possible with a cold-blooded animal when the ester itself is solid at the water temperature (even when mixed with 20 % of eel fat) and the acid, of course, would be quite hard. There was no visible evidence of bad absorption of the acid, no large fatty stools being observed, such as Cox [1933] noticed with rats fed with ethyl palmitate or stearate. It is clear from Table 1 that the amount of palmitic acid finally appearing in the depot fat is only a small fraction of that fed, and this result is to be expected by analogy with earlier work on eels fed on herring [Lovern, 1938]. The remainder of the ingested fat is presumably utilized for energy purposes, and it is notable that these eels were the only ones to maintain their weight throughout the experiment.

The incorporation of this extra acid in the depot fat will, other things being equal, cause a fall in the proportions of all other constituents and the figure for hexadecenoic acid in the untreated eel fat would fall to 8·1 % if sufficient palmitic acid was incorporated to raise the total palmitic acid content to 25·7 %. If the total saturated acid content of the fat is considered, rather than palmitic acid alone (it has been indicated that the apparent rise in myristic acid is at the expense of palmitic acid, and in any case it is the total content of saturated acids which would provide the stimulus to dehydrogenation in the depot fat), the corrected figure for the hexadecenoic acid content of the untreated eel fat becomes 7·8 %. Obviously the content of all the other unsaturated acid groups would also fall. Actually, however, the hexadecenoic figure has not fallen but the content of the other unsaturated acids has fallen, as would be expected.

The results therefore are just as would be predicted by the theory of dehydrogenation of palmitic acid under suitable stimulus, but the small extent of the changes makes their evidence uncertain. A difference between 9·1 and 7·8 % of hexadecenoic acid is probably just outside the limit of error to be expected by an experienced worker, but there remains the question of normal variation in eel fat composition. Only two normal eel fat analyses are available, and, as already mentioned, the agreement there was close. Each analysis represented many fish but there seems no reason why small variations should not be expected from season to season, owing to differences in food supply, etc. If all the fats from eels fed on fat-poor diets are considered [Lovern, 1938], quite considerable variations are shown, but the average figure for hexadecenoic acid for all these samples is below 9 % and it seems unlikely that this figure should be exceeded in the fat from normal eels taken from these particular waters (the estuary of the River Dee). The one sample which did have a higher content of this acid was abnormal in other respects (high content of palmitic acid and unusual content of fat in the fish) suggesting that this batch of fish had had an unusual dietary history. Nevertheless, in view of the combined experimental error and natural variation, it would be unsafe to regard the results as a proof of the theory, although they are absolutely in accord with it.

Turning to the second experiment in the feeding of mixed unsaturated esters, such a diet should lower the content of palmitic and, to a very slight extent, raise that of hexadecenoic acid. In effect a slight fall in palmitic acid has taken place (the figure of 16·0 % being the lowest ever encountered in any eel fat,

normal or otherwise) and the hexadecenoic acid figure, instead of rising, has also fallen.

These results are also just as would be predicted on the theory of hydrogenation of hexadecenoic acid under suitable stimulus, part of the ingested acid being hydrogenated (either during absorption or in the depots) in an attempt to maintain the normal content of saturated acids. The apparent entire absence of tetradecenoic acid is also of interest and again suggests a hydrogenation process. However, the previous remarks regarding combined experimental error and natural variation apply here also.

Considering all the results of both series of experiments it may be said that they are all in accordance with the theory and also that the extent of the changes has varied in the appropriate way with the intensity of feeding. It seems rather unlikely that experimental error and natural variation have in all cases caused such a situation and the writer believes that whilst more certain data are highly desirable, these results do provide additional support for the theory of interconvertibility of certain saturated and unsaturated acids.

Summary

In two series of feeding experiments, ethyl myristate, ethyl palmitate and the ethyl esters of the mixed unsaturated acids of eel fat have been fed to eels. Rather surprisingly, good absorption on the palmitate diet was indicated.

The diets produced certain modifications in the composition of the eel body fats, but apart from a noteworthy increase in the content of palmitic acid when ethyl palmitate was fed, the changes were of too small an order to be differentiated with certainty from combined experimental error and natural variation.

The consistency of these changes, however, increases the probability that they were genuine. Feeding ethyl myristate caused a rise in tetradecenoic acid, ethyl palmitate caused a rise in hexadecenoic acid and the mixed unsaturated esters gave results suggesting hydrogenation of hexadecenoic acid.

The results, therefore, whilst not conclusive, afford additional support for the theory of the interconvertibility by fish of certain saturated and unsaturated acids.

The work described above was carried out as part of the programme of the Food Investigation Board, and is published by permission of the Department of Scientific and Industrial Research.

REFERENCES

Banks & Hilditch (1931). *Biochem. J.* **25**, 1168.
—— —— (1932). *Biochem. J.* **26**, 298.
Cox (1933). *J. biol. Chem.* **103**, 777.
Hilditch (1937). *Analyst*, **62**, 250.
Longenecker (1939). *J. biol. Chem.* **130**, 167.
Lovern (1937). *Biochem. J.* **31**, 755.
—— (1938). *Biochem. J.* **32**, 1214.
Rittenberg & Schoenheimer (1937). *J. biol. Chem.* **117**, 485.
Schoenheimer & Rittenberg (1936). *J. biol. Chem.* **113**, 505.

87. THE LIPINS OF THE EARTHWORM

By JOHN ARNOLD LOVERN

From the Torry Research Station, Aberdeen, of the Department of Scientific and Industrial Research

(*Received 1 April 1940*)

THE majority of animal fats examined in detail have been from vertebrates. In connexion with the feeding experiments described in the preceding paper, a knowledge of the composition of the mixed fatty acids of earthworms was desirable and a large quantity of worms was collected over a considerable period and kept in cold storage until sufficient was available for fatty acid analyses. In view of the unexpectedly complex mixture of acids found, a second, larger quantity of worms was collected and a second analysis carried out, which confirmed in every respect the first conclusions.

EXPERIMENTAL

In the first experiment, 25,700 g. of earthworms gave 310 g. (1·2 %) of ether-soluble matter, which was separated into 175 g. (56·4 %) of acetone-soluble "fat" (fraction A) and 135 g. of acetone-insoluble "phosphatides" (B). In the second experiment 54,450 g. of worms gave 697 g. of ether-extract (1·3 %) which gave 467 g. (67 %) of acetone-soluble "fat" (A_1) and 230 g. of "phosphatides" (B_1).

All these fractions were very dark in colour and all contained material other than fat. Fraction A gave 75·5 g. acids and 90·0 g. of unsaponifiable matter. The loss in weight largely represents water-soluble material, of unknown nature, which often accompanies crude fat extracts. Fraction A_1 gave 223 g. acids and 170 g. of unsaponifiable matter. The unsaponifiable material was mainly of a sterol-like nature [cf. Bock & Wetter, 1938] and is unusual in containing about 23 % of ergosterol. Along with these sterols there was a viscous liquid fraction. It proved unusually difficult to extract the unsaponifiable matter completely and even after 25 extractions of the aqueous soap solution with large volumes of ether several g. of unsaponifiable matter remained. This proved partly volatile during the subsequent vacuum distillation of the methyl esters, and many fractions had to be freed from traces of it before reliable equivalents could be obtained.

The acids were purified by means of light petroleum, which removed a few g. of insoluble acids. The petrol-soluble acids were subjected to lead salt-alcohol separation and the fractions esterified (by the usual process with methyl alcohol and sulphuric acid). Esterification, particularly of the solid acids, proved very incomplete, and the unesterified material, a black, sticky mass, is of unknown nature and probably polybasic. That recovered from the solid acids (of A_1) had I.V. 44·5 and sap. equiv. 240·2, whilst that from the liquid acids (A_1) had I.V. 92·0 and sap. equiv. 617·6. In calculating the final composition of the acids, the proportions of esters, rather than acids, finally resulting from the lead salt separation was used, since the non-esterifiable acids cannot be of ordinary fatty nature and it was desired to exclude them from the final results.

The "phosphatide" fractions were similarly treated but proved to contain very large proportions of non-fatty material. Fraction B gave 40 g. of petrol-soluble acids, 28 g. of petrol-insoluble acids and 11·5 g. of unsaponifiable matter. There was a large loss of water-soluble matter. Fraction B_1 gave 31·5 g. of petrol-soluble acids, 9 g. of petrol-insoluble acids and 69 g. of unsaponifiable matter. There are obviously quantitative differences between these two crude fractions. The unsaponifiable matter from B was a gummy solid, whilst that from B_1 was a liquid, both of unknown nature. The petrol-insoluble acids (from B_1) had I.V. 65·24 and sap. equiv. 288·4. Once again there was a considerable content of non-esterifiable acids. The acids from B_1, in view of the small amounts of esters finally resulting, were not submitted to fractional distillation, as the results could not have been so accurate as those already obtained for B.

Table 1. *Compositions of fatty acids of earthworm lipins (weights %)*

	Saturated acids					
Fraction	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	$C_{>18}$
A	—	6	6	8	11	1
A_1	1	4	6	6	13	2
B	—	3	2	5	8	3

	Unsaturated acids						
Fraction	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	$C_{>22}$
A	2 (−2·0 H)	7 (−2·7 H)	Nil ?	20 (−3·9 H)	21 (−6·8 H)	10 (−? H)	8 (−? H)
A_1	3 (−2·0 H)	4 (−2·9 H)	3 (−2·8 H)	22 (−4·2 H)	26 (−6·5 H)	2 (−? H)	8 (−? H)
B	1	1	Nil ?	4 (−3·7 H)	42 (−4·9 H)	17 (−4·4 H)	14 (−? H)

The lead salt separation was unusually inefficient in all cases, much higher saturated acid (e.g. stearic) going into the liquid acid fraction whilst, at the same time, much lower polyethylenic acid (e.g. C_{14}) went into the solid acid fraction. The reason for this is quite obscure. It was necessary to oxidize a great many of the fractions by the permanganate-acetone method [Harper *et al.* 1937] before their compositions could be calculated.

The compositions of the esterifiable fatty acid mixtures are given in Table 1. In view of the unusual complexity of these lipins and the difficulties encountered during the analysis (e.g. the presence of volatile unsaponifiable matter), these results cannot be regarded as of the usual order of accuracy. In addition to the esterifiable acids, the crude acid mixtures contained 17, 10 and 54% of "non-esterifiable" (and petrol-insoluble) acids for A, A_1 and B respectively. B_1 acids contained 72% of such non-fatty acids.

Discussion

It will be seen from Table 1 that the two analyses for the acetone-soluble fraction are in good agreement considering the difficulties mentioned. The phosphatide acids are in general of a similar nature, but contain rather less of saturated acids and much less C_{18} unsaturated, whilst the contents of higher unsaturated acids are much larger. In each case the saturated acids with more than 18 carbon atoms had a mean equiv. wt. of about 350, and were probably a mixture. The unsaturated acids with more than 22 carbon atoms were also presumably a mixture and had mean equiv. wt. of over 400.

Points of interest in these unusual acid mixtures include the presence of lower mol. wt. derivatives, both saturated and unsaturated, the very low content of hexadecenoic acid, the high ratio of stearic to palmitic acid and the high degree of average unsaturation of the C_{14} and C_{18} acid groups. It did not prove possible to obtain reliable figures for the average unsaturation of each group of C_{22} and $C_{>22}$ acids, but all were certainly polyethylenic.

In view of the high content of unsaponifiable matter in the acetone-soluble fractions it seems doubtful whether much ordinary triglyceride could have been present, most of the fat presumably being sterol, etc., esters.

The unusual nature of these fatty acid mixtures indicates the desirability of obtaining data for other classes of invertebrates which have not so far been examined.

SUMMARY

The acids occurring in the acetone-soluble and acetone-insoluble fractions respectively of earthworm lipins have been examined. In addition to acids of unknown (possibly non-fatty) nature, these contain fatty acids ranging from C_{10} to $C_{>22}$, including unsaturated C_{12} acids. Ordinary triglyceride cannot be an important constituent in view of the high content of sterols.

The work described above was carried out as part of the programme of the Food Investigation Board, and is published by permission of the Department of Scientific and Industrial Research.

REFERENCES

Bock & Wetter (1938). *Hoppe-Seyl. Z.* **256**, 33.
Harper, Hilditch & Terleski (1937). *J. Soc. chem. Ind., Lond.*, **56**, 310 T.

88. ESTIMATION OF NICOTINIC ACID IN ANIMAL TISSUES, BLOOD AND CERTAIN FOODSTUFFS

1. METHOD[1]

By E. KODICEK

From the Nutritional Laboratory, University of Cambridge and Medical Research Council

(*Received 29 March 1940*)

SINCE the importance of nicotinic acid as the blacktongue- and pellagra-preventing vitamin and as an essential part of coenzyme systems—the phosphopyridine nucleotides—has been established, a reliable method for its estimation in various substances is needed; this should help to deepen the understanding of its physiological and biochemical role in the living organism.

Methods for the estimation of nicotinic acid in tissues and foodstuffs

The following reagents have been proposed for the estimation of nicotinic acid in animal tissues, blood or foodstuffs:

(1) 2:4-Dinitrochlorobenzene was used by Karrer & Keller [1938, 1, 2; 1939] and by Vilter *et al.* [1938]; it gives a colour reaction with pyridine derivatives [Vongerichten, 1899; Zincke, 1904]. This method is much less sensitive than the following and much more laborious.

(2) Aniline was used by Swaminathan [1938, 1, 2], Kringstad & Naess [1938; 1939], Pearson [1939], Ritsert [1939]. It produces a colour reaction of the type described by König [1904] and by Strafford & Parry Jones [1933], i.e. pyridine derivatives react with cyanogen bromide plus an aromatic amine to yield a coloured substance.

(3) A similar procedure was followed by Bandier & Hald [1939] and by Bandier [1939], who however used metol instead of aniline.

(4) v. Euler *et al.* [1938] described a modification of Barta's [1935] method in which naphthylamine is the reagent employed.

(5) In blood, nicotinic acid has also been estimated quantitatively by its growth-promoting action on various micro-organisms [e.g. Querido *et al.* 1939].

Use of p-*aminoacetophenone and comparison with metol and aniline*

In the work described in this paper *p*-aminoacetophenone, introduced by Harris & Raymond [1939, 1, 2], was used as the aromatic amine for the König reaction. This method seems to be more satisfactory than any of the others previously described. Some of the advantages of this substance have already been stated by Harris & Raymond [1939, 2].

The amount of colour developed in the cyanogen-amine reaction depends on many variable factors, which are not absolutely controllable, hence the use of a standard reference curve cannot be recommended. This difficulty was successfully circumvented by the procedure described by Harris & Raymond (see below).

Aniline and metol give less colour, as will be seen in Fig. 1. With increasing amounts of the aromatic amine the colour increases to a certain maximum.

[1] Communicated to the Biochemical Society, 9 December 1939 [Kodicek, 1939].

p-Aminoacetophenone developed an intense colour even with 10 μg. nicotinic acid in 15 ml., and when as little as 0·2 ml. of amine solution (10 %) was used. The same intensity was reached as a maximum only with as much as 5 ml. of aniline (2 %), and the maximum intensity of colour with 10 ml. of *ca.* 5 % metol was even lower. Also because of their relative insolubilities a larger volume of the reagents has to be taken when aniline or metol are employed for work with animal extracts, and this unavoidable dilution further diminishes the sensitivity. It was quite obvious therefore that *p*-aminoacetophenone was more suitable for our purpose than other amines, many of which had already been examined by Harris & Raymond.

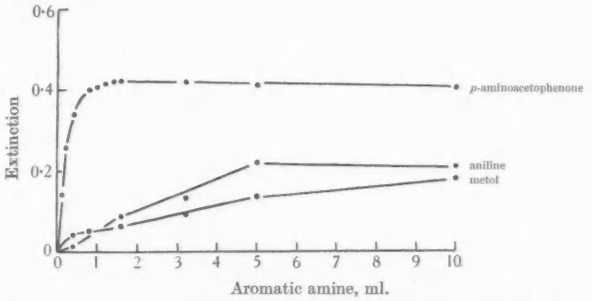


Fig. 1. Comparison of colour intensity of different aromatic amines (10 μg. of nicotinic acid used).

In the work described in this paper the various factors influencing the reaction have been checked step by step, so as to eliminate possible errors as far as possible, and quantitative studies have been made of the conditions affecting the accuracy of the results.

Principle of the method used

The material is first extracted or hydrolysed as it stands with 8 % NaOH or H_2SO_4 for 1 hr. By this treatment derivatives of nicotinic acid such as amides, pyridine nucleotides and possibly others, are converted into free nicotinic acid. Other substances are next precipitated by the addition of an excess of 96 % ethyl alcohol, leaving the nicotinic acid in solution. The reaction is cautiously brought to *p*H 6 with conc. HCl, with the use of an external indicator and with $NaHCO_3$ as a buffer. The solution is made up to a fixed volume. Three samples are taken to circumvent the use of a standard reference curve, one for the unknown, and two for the unknown to which have been added known amounts of nicotinic acid; there is also a blank. These are heated with cyanogen bromide and later treated with acidified *p*-aminoacetophenone. From the 3 samples 3 colour readings are taken in the Pulfrich photometer, and as the reaction has been shown to obey Beer's law the amount of nicotinic acid in the unknown can be calculated, all 3 results lying on a straight line [see Harris & Raymond, 1939, 2; Kodicek, 1939].

Quantitative study of factors influencing the reaction

(*a*) *Methods of extraction.* For animal tissues, three methods of extraction, called *a*, *b* and *c* in Table 1, can be used, all of which give, so far as has been found, the same final result for the total amount of nicotinic acid present. Extraction

Table 1. *Alternative methods of extraction*

Extraction method ...	Nicotinic acid found, μg. per g. fresh weight				
	a	*b*	*c*	*d*	*e*
Liver (ox)	186	187	182	—	90
Muscle (ox)	40	40	40	—	11
Goldberger diet*	17	26	6	20	—
Yellow maize meal (sample D)	41	45	8	37	—
,, (sample C)	12	27	6	—	—
,, (sample B)	18	33	—	—	—

Extraction *a*. Minced tissue suspended in water and hydrolysed with NaOH or H_2SO_4.

Extraction *b*. Minced tissue suspended in EtOH and hydrolysed with NaOH or H_2SO_4.

Extraction *c*. Extracted with water at 100°; extract subsequently hydrolysed with NaOH.

Extraction *d*. Residuum after water extraction *c* hydrolysed with NaOH and estimated for nicotinic acid.

Extraction *e*. Extracted with 96% EtOH and extract subsequently hydrolysed with NaOH.

* Goldberger diet; yellow maize meal 4000, pea meal 500, extracted casein A/E (Glaxo) 600, $CaCO_3$ 30, NaCl 100, cottonseed oil 300.

during hydrolysis with NaOH has been the method actually used in this and the following paper. For animal tissues extraction by boiling water is equally effective, indicating that all the nicotinic acid present is soluble in water, or rapidly liberated by boiling. For cereals the question of extraction is more complicated. It will be seen from Table 1 that extraction of cereals with water gave distinctly lower results than the other methods. It is not yet certain which type of extraction gives the real content of biologically active nicotinic acid. Some varieties of yellow maize meal, as in Table 1, apparently contained very high concentrations of nicotinic acid. However, L. J. Harris and I (unpublished) found that these samples of maize were as inactive in blacktongue experiments as white maize which gave a lower colour value in the chemical test. It seems probable therefore that the watery extract (method *c*) contains the "true" or "active" nicotinic acid, the concentration of which would thus be very low. It would in this respect behave like the active nicotinic acid in animal tissues which is readily extractable by water. Thus the total concentration of substances derived from cereals which give the cyanogen-amine reaction when estimated by direct hydrolysis either in water (method *a*) or in alcohol (method *b*), does not presumably represent the "true" or "active" nicotinic acid. This point has to be left open for investigation in further work which is now in progress; it will be mentioned again in the following paper [Kodicek, 1940].

Hydrolysis in ethyl alcohol (method *b*, Table 1) was of some help on occasions when it was preferable to use water as little as possible in order to obtain clear, non-turbid solutions. The fourth method of extraction (*e*)—precipitation with ethyl alcohol before hydrolysis—yielded lower results, which are not due to imperfect extraction, but which make it possible to distinguish an "alcohol-soluble fraction". Whether this fraction can be explained as a partial precipitation of certain nicotinic acid derivatives or as a selective precipitation of some special fraction has not yet been determined.

(*b*) *Time necessary for hydrolysis with 8% NaOH.* As shown in Table 2, unhydrolysed nicotinamide gives only 20% of the colour of the equivalent amount of nicotinic acid. But after hydrolysis for only 5 min. it reaches the maximum colour, being converted into nicotinic acid. The same is true of the nicotinic acid derivatives in liver and muscle; the coenzymes are very easily

Table 2. *Time necessary for hydrolysis with 8% NaOH*

	Nicotinic acid, μg./g. fresh weight				
		Hydrolysed			
	Unhydrolysed	5 min.	30 min.	90 min.	180 min.
Liver, ox (sample B)	0	113	—	97	—
,, (sample B)	20	—	160	120	150
Muscle, beef	10	—	40	40	40
Nicotinamide: % recovery	20	104	—	104	93
Nicotinamide boiled in water for 15 min.: % recovery	38	—	—	—	—

hydrolysed. The "unhydrolysed alcoholic fraction" of liver and muscle was obtained by precipitating the watery extract with excess of ethyl alcohol (see Table 2, and Table 8 in the following paper), and the solution was estimated without preceding hydrolysis. It is assumed that by this procedure the free nicotinic acid and one-fifth of the free amide are estimated.

(*c*) *Concentration of NaOH.* Results obtained after hydrolysis for 1 hr. are summarized in Table 3 For animal tissues 4% NaOH was effective; nevertheless, for the work described in this paper a final concentration of 8% NaOH has been used. As nicotinic acid is very stable, even to prolonged treatment with alkali, there is no objection to the use of the more concentrated solution of NaOH. The recovery of cozymase was calculated from the nicotinic acid found after hydrolysis compared with figures obtained from the direct spectrographic estimation of the cozymase itself.[1]

Table 3. *Hydrolysis with NaOH for 1 hr.*

	Nicotinic acid, μg./g. fresh weight Final concentration of NaOH	
	4%	8%
Muscle, beef	40	42
Cod	30	28
Salmon	95	82
Whole wheat	26	30
Whole flour	13	15
Cozymase (Green): % recovery	82	89 impure (11% purity)

Table 4. *Hydrolysis with H_2SO_4 for 1 hr.*

	Nicotinic acid, μg./g. fresh weight		
	Final concentration of H_2SO_4		Hydrolysis with 8% NaOH
	4%	8%	
Muscle, beef	10	40	40
Whole meal	26	42	30
White flour	—	15	15

(*d*) *Concentration of H_2SO_4 for hydrolysis.* Table 4 gives the results obtained in hydrolyses with different concentrations of H_2SO_4. A final concentration of 4% gives low results. A concentration of 8%, however, gives similar results to

[1] I am indebted to Sir Charles Martin for placing at my disposal a preparation of the cozymase which he had received from Prof. v. Euler, and to Dr D. E. Green for other specimens and also for the spectrographic estimation.

those obtained by hydrolysis with 8% NaOH. As trigonelline, according to Karrer [1938], may be converted by hydrolysis with acid into nicotinic acid, it seemed to be preferable, for the sake of specificity, to hydrolyse with NaOH.

(*e*) *Precipitation of other substances.* Trichloroacetic acid, 20%, was tried for the preparation of an extract (see Table 5). Nicotinic acid in water, or added to

Table 5. *Precipitation with 20% trichloroacetic acid*

	Total nicotinic acid found	Recovery of added nicotinic acid	
	μg.	μg.	%
10 ml. serum + 5 ml. trichloroacetic acid	0·0	—	—
+ 20 μg. nicotinic acid	20·0	20·0	100
+ 150 μg. nicotinic acid	146·0	146·0	97
25 ml. urine	148·0	—	—
+ 5 ml. trichloroacetic acid + 30 μg. nicotinic acid	180·0	32·0	107
+ 100 μg. nicotinic acid	243·0	95·0	95
20 μg. nicotinic acid in 4% trichloroacetic acid	20·0	20·0	100

serum and urine, could be recovered quantitatively. Serum itself was found to contain no nicotinic acid. In one experiment 10 ml. of serum (horse) plus added nicotinic acid were treated with 5 ml. of 20% trichloroacetic acid; 10 ml. distilled water were added, the product was centrifuged and the supernatant liquid then hydrolysed. The colour faded slowly however and was less intense (cf. also Table 5*a*); trichloroacetic acid used after hydrolysis was also unsatisfactory.

Table 5*a*. *Stability of colour in trichloroacetic acid*

	Time min.	Extinction
20 μg. nicotinic acid in 4% trichloroacetic acid	0	0·33
	10	0·29
	20	0·28
	40	0·27
	60	0·26
	150	0·16

On the other hand, precipitation with excess of 96% ethyl alcohol (9 parts), acetone or amyl alcohol gave better results. With ethyl alcohol, nicotinic acid could be recovered quantitatively either in pure solution (e.g. Table 6) or added

Table 6. *Precipitation with 96% ethyl alcohol*

	Amount taken	Nicotinic acid found, μg.	
	μg.	In water	In ethyl alcohol
Nicotinic acid	100	99 (±2·0)	103 (±1·3)
Nicotinamide	100	102 (±3·2)	105 (±3·5)
Urine (dog)	—	1·2 μg. per ml.	1·2 μg. per ml.
Urine (human)	—	1·6 μg. per ml.	1·8 μg. per ml.

Figures in brackets give the standard error of the mean.

to various tissue extracts (Table 10). Clear urine yielded the same results whether ethyl alcohol was used or not. The intensity and stability of colour were the same as in water. Thus with ethyl alcohol no interfering substances are introduced and the other difficulties alluded to above are obviated. The final con-

centration of alcohol must be above 80 %. Below this some tissue extracts often get turbid. The following results were noted with extracts from liver tissues.

Concentration of ethyl alcohol %	Result
78	Solution turbid
81	Solution clear
84	Solution clear

(*f*) *Influence of* p*H during treatment with CNBr.* As Harris & Raymond [1939, 2] have explained, the concentration of H ions is most important; this was found also by other authors who used aniline. If the *p*H, however, is adjusted with or without buffer within the range of 5·5–7·5, a reproducible, non-varying colour of maximum intensity may be obtained (Fig. 2).

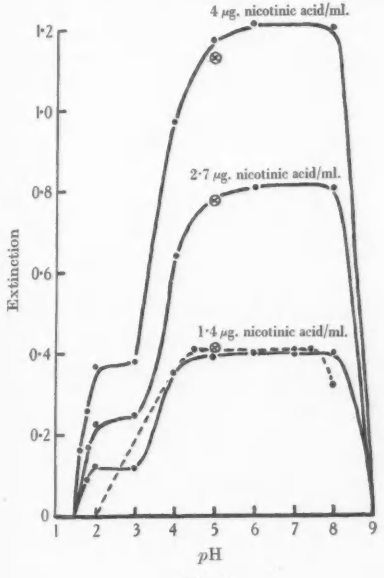


Fig. 2.

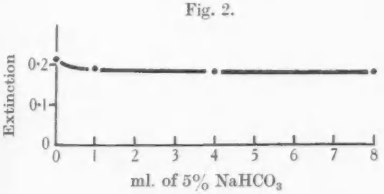


Fig. 3.

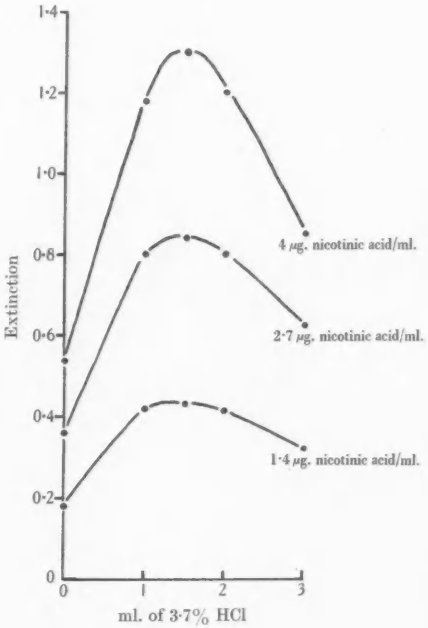


Fig. 4.

Fig. 2. Influence of *p*H. 3 cm. cells in step-photometer; —— *p*H adjusted with HCl; - - - - *p*H adjusted with phosphate buffer, 0·1 *M*; ⊗ *p*H adjusted with acetate buffer, 0·1 *M* at *p*H 5.

Fig. 3. Influence of salts. Nicotinic acid 1 μg. per ml. Increasing amount of 5 % $NaHCO_3$.

Fig. 4. Influence of acidification after addition of 0·2 ml. of *p*-aminoacetophenone.

(*g*) *Influence of salts.* The influence of salts is less marked. $NaHCO_3$ in increasing concentration causes a very slight fading (Fig. 3). Acetate buffer (Fig. 2) 0·1 *M* at *p*H 5 did not interfere with the development of the colour, as was found by v. Euler *et al.* [1938] to be the case when other aromatic amines were used. Glacial acetic acid in high concentration, however, when used for preservation of urine, prevented the development of any colour at all.

(*h*) *Use of cyanogen bromide.* (1) *Preparation of CNBr.* It was found to be of importance to prepare the substance in ice-cold solutions [Kulikow & Krestowosdwigenskaja, 1930]. The reproducibility of the results was thereby increased.

(2) The heating to 70–80° is necessary for the reaction. Without heating no colour developed. If CNBr were added together with *p*-aminoacetophenone and the mixture then heated, no colour was produced.

(3) *Different amounts of CNBr.* There was no change in the intensity of the colour when CNBr was used in amounts varying from 0·5 ml. to 4 ml. in 15 ml. of solution (e.g. Table 7).

Table 7. *Different amounts of CNBr added to 10 µg. nicotinic acid and 0·2 ml. 10% p-aminoacetophenone*

Amount of CNBr, ml.	Extinction	Amount of CNBr, ml.	Extinction
0·0	0·00	2·0	0·20
0·5	0·20	4·0	0·20
1·0	0·20	8·0	0·18

(*i*) *Development of colour. Effect of acidification on colour after adding* p-*aminoacetophenone.* Acidification upon the addition of the *p*-aminoacetophenone is another important factor (Fig. 4). The optimum amount under the conditions described for Fig. 4 was found to be 1·2 ml. of 3·7% HCl. The *p*-aminoacetophenone was prepared as follows: to 5 g. *p*-aminoacetophenone 14 ml. of 10% HCl were added, and the volume was made up to 50 ml. with water. Later, however, it was found more convenient to add the *p*-aminoacetophenone and the optimum amount of HCl together in the same solution, and the procedure then adopted was to use 0·4 ml. of an acidified solution of *p*-aminoacetophenone. The mixed solution of *p*-aminoacetophenone and HCl was prepared as follows: to 5 g. *p*-aminoacetophenone 30 ml. of 32% HCl were added and the volume was made up to 100 ml. with water. There was no difference in the intensity of the colour whether the HCl was added with the *p*-aminoacetophenone or after it.

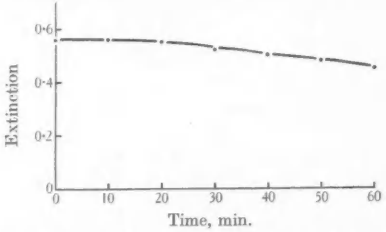


Fig. 5. Time curve after adding acidified *p*-aminoacetophenone (0·4 ml.) to 1·4 µg. of nicotinic acid per ml.

(*j*) *Time after addition of acidified* p-*aminoacetophenone.* The colour is fairly stable for 20–30 min. if kept in the dark (Fig. 5). The estimation was actually performed during the first 15 min. after the addition of the amine reagent.

Colour curve

The extinction curve of the coloured solution as determined in the step-photometer declines from violet to red (Fig. 6). For the estimations of nicotinic acid the blue filter No. 47 of the step-photometer has been used. Filter No. 43 was found to be too dark.

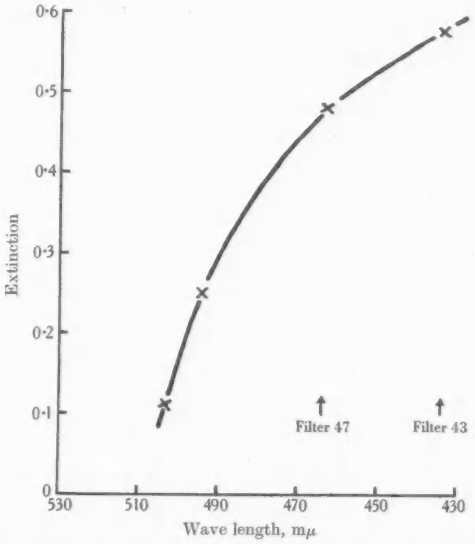


Fig. 6. Extinction curve. Nicotinic acid, 2 μg. per ml.; 1 cm. cells in step-photometer.

Specificity

Numerous amino-acids and other substances unrelated to pyridine were investigated and none gave a colour reaction (Table 8). Among the pyridine derivatives which were available at the time, adermin, trigonelline and quinolinic acid were found to give no colour. Pyridine, α-aminopyridine, nipecotic acid and nicotinic acid-*N*-diethylamide (coramine) gave some colour. But if no more than 20 μg. in 15 ml. of each substance were used, only with nicotinic acid-*N*-diethylamide was the colour measurable, being of the same intensity as that of nicotinic acid, into which it is converted upon hydrolysis. This substance is also biologically active, whereas the others are said to be inactive [Woolley *et al.* 1938]. It is interesting to notice how these substances differ, according as to whether they are estimated before or after hydrolysis. Nicotinamide gives only 20 % of its maximum colour if unhydrolysed; but, in contrast, nicotinic acid-*N*-diethylamide gives a more intense colour without hydrolysis. It seems that the side chain in the β-position is of some importance for the reaction. The unoccupied neighbouring carbon atoms in the α-position must be free to take part in the opening of the pyridine ring. It would appear from this suggestion that some pyridine derivatives fulfilling these conditions might give the reaction without being active in blacktongue or pellagra. This point has to be considered in the estimation of nicotinic acid in cereals.

Table 8. *Specificity of reaction*

(1) Miscellaneous substances

Negative. *l*-Cystine, cysteine, glutathione, glycine, *d*-alanine, *d*-lysine hydrochloride, *d*-arginine carbonate, *l*-leucine, *iso*leucine, histidine dichloride, adenine, guanidine hydrochloride, *d*-*l*-valine, *l*-asparagine, glutamic acid, aspartic acid, guanylic acid, urea, β-hydroxyproline, *l*-proline, nucleic acid, uric acid, adenosine, guanosine, inulin, inositol, xylose, protocatechuic acid, 2-amino-4-methylthiazole-5-carboxylic ester, 3-benzyl-2:3-dimethylthiazoloniumbromide, aneurin, tyrosine, thyroxine, creatinine, isoxazolecarboxylic acid, and pyrrole if hydrolysed.

(2) Pyridine derivatives

Negative. Adermin, trigonelline, quinolinic acid.
Colour developed. Pyridine, α-aminopyridine, nipecotic acid, nicotinic acid-*N*-diethylamide.

Extinction values with 20 μg. of pyridine derivatives

	Extinction	
	Hydrolysed	Unhydrolysed
Nicotinic acid	0·48	0·48
Nicotinamide	0·48	0·10
Nicotinic acid-*N*-diethylamide	0·47	0·70
Pyridine	0·0	0·0
α-Aminopyridine	0·0	0·0
Nipecotic acid	0·0	0·0
Adermin	0·0	0·0
Trigonelline	0·0	0·0
Quinolinic acid	0·0	0·0

(3) Solvents

Negative. Ethyl alcohol, chloroform, sulphur-free toluene, trichloroacetic acid, acetone, ethyl acetone, amyl alcohol.
Slight colour (due to impurities present). Toluene, *iso*butyl alcohol, benzene, propyl alcohol.

Some of the solvents, if impure, may give a colour. Impure toluene gives a positive reaction but repurified "sulphur-free" toluene is negative and can therefore be used for the preservation of urine or other biological material.

Sensitivity

As little as 1–2 μg. of nicotinic acid per g. of material can generally be detected with *p*-aminoacetophenone. This appears to be about 3–5 times as sensitive as aniline and metol.

Standard reference curve

Such a curve (Fig. 7) can probably be used only for any one given material, and then only when the conditions already described are regularly fulfilled. The standard reference curve has the equation:

$$\mu g. \text{ nicotinic acid in aliquot sample} = 47A,$$

where A is the extinction value of the unknown. The Pulfrich step-photometer was used, with 3 cm. cells and filter No. 47. For a given substance, for example muscle (Table 9), the agreement is good. If, however, a different material is taken the results from the standard reference curve and from the experimentally extrapolated curve (see above) may differ appreciably from one another. In Fig. 8 readings obtained on a great variety of material have been collected for comparison. The values obtained from the extrapolated curve, in the manner suggested by Harris & Raymond [1939, 2], using additions of known amounts of

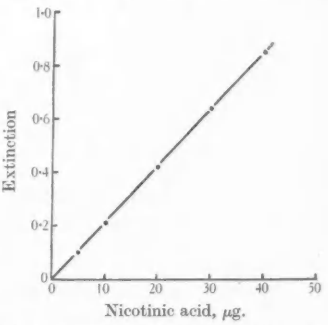


Fig. 7. Standard reference curve for nicotinic acid. 3 cm. cells in step-photometer, 0·4 ml. of *p*-aminoacetophenone.

Table 9. *Reproducibility of results*

Nicotinic acid found per g. of muscle in separate determinations on same specimen

(1) Method of extrapolation	(2) Comparison against standard reference curve for each single reading
μg.	μg.
40·0	40·0
40·0	40·0
40·0	42·5
35·0	32·5
45·0	47·5
40·0	45·0
52·5	58·5
42·5	47·5
40·0	45·0
45·0	47·5
47·5	47·5
42·5	45·0
42·5 (± 1·3 standard error of the mean)	44·8 (± 1·8 standard error of the mean)
Standard error of an individual result ± 4·52	± 6·24

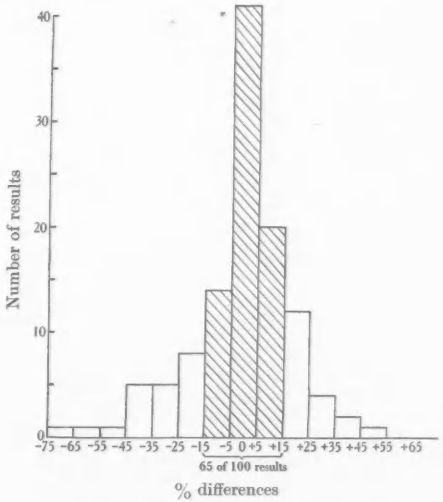


Fig. 8. Distribution of % differences between results from extrapolated curve and standard reference curve.

nicotinic acid to obtain a straight line, were accepted as the true results. The percentage differences of the results from those given by the standard reference curve were plotted as a histogram (Fig. 8). Out of 115 results 75 (65 %) lay within the range of ± 15 % difference. One of three results would have a greater error than this range. Therefore it seems preferable to use the extrapolated curve. The standard reference curve can be used only as a check on the reliability of the result.

Statistical accuracy of the method

Table 9 shows the results of repeated determinations on the same material (muscle). It will be seen that there is little variation. The standard error of an individual observation by the method of extrapolation is 4·52 mg. or 10·6 %.

Recovery of nicotinic acid and amide

The recovery of nicotinamide and nicotinic acid is complete either in solution in ethyl alcohol (with a standard error of ±3·5 % and ±1·3 % respectively), or when added to various substances (Table 10).

Table 10. *Recovery of added nicotinic acid and amide*

Description	Amount taken μg.	No. of esti-mations	Amount found μg.	Recovery %
Nicotinic acid in ethyl alcohol	50·0	15	51·5 (Mean value)	103 (±1·3 standard error)
Nicotinamide in ethyl alcohol, hydrolysed	50·0	16	52·5 (Mean value)	105 (±3·5 standard error)
Nicotinamide in ethyl alcohol, unhydrolysed	50·0	3	10·0 (Mean value)	20
Nicotinic acid added to:				
Serum	50·0	2	48·5 (Mean value)	97
Yeastrel	50·0	3	49·5 (Mean value)	99
Egg white	50·0	1	50·0	100
Marmite	50·0	2	57·5 (Mean value)	115
Muscle	50·0	2	54·0 (Mean value)	108
Milk	50·0	2	55·0 (Mean value)	105

SUMMARY

The procedure is discussed for the estimation of nicotinic acid in biological material by means of the cyanogen-*p*-aminoacetophenone method of Harris & Raymond. A quantitative study has been made of the various factors influencing the accuracy of the results: e.g. alternative methods of extraction, rate of hydrolysis, removal of interfering substances by means of ethyl alcohol, effects of changes in *p*H and of the final acidification. The specificity of the colour reaction is discussed. As little as 1–2 μg. of nicotinic acid can be detected in 1 g. of material, and in control tests quantitative recovery of added nicotinic acid was effected. This method is more sensitive than those in which metol and aniline are used.

In certain animal tissues nicotinic acid seems to be present bound to substances which are easily extracted by boiling water but which are partly precipitated by ethyl alcohol. In some cereals only a small fraction of the chromogen behaves like the nicotinic acid of animal tissues, i.e. is readily extracted by boiling water. It is believed that only this fraction (or a portion of it) is the "true" or "active" nicotinic acid; otherwise yellow maize which is known to be deficient would have a high concentration of nicotinic acid.

REFERENCES

Bandier (1939). *Biochem. J.* **33**, 1130.
—— & Hald (1939). *Biochem. J.* **33**, 264.
Barta (1935). *Biochem. Z.* **277**, 412.
v. Euler, Schlenk, Heiwinkel & Högberg (1938). *Hoppe-Seyl. Z.* **256**, 208.
Harris & Raymond (1939, 1). *Chem. Ind.* **58**, 652.
—— —— (1939, 2). *Biochem. J.* **33**, 2037.
Karrer (1938). Organic chemistry. Amsterdam.
—— & Keller (1938, 1). *Helv. chim. Acta*, **21**, 463.
—— —— (1938, 2). *Helv. chim. Acta*, **21**, 1170.
—— —— (1939). *Helv. chim. Acta*, **22**, 1292.
Kodicek (1939). *Chem. Ind.* **58**, 1088.
—— (1940). *Biochem. J.* **34**, 724.
König (1904). *J. prakt. Chem.* **69**, 105; **70**, 19.
Kringstad & Naess (1938). *Naturwissenschaften*, **26**, 709.
—— —— (1939). *Hoppe-Seyl.* **260**, 108.
Kulikow & Krestowosdwigenskaja (1930). *Z. anal. Chem.* **79**, 452.
Pearson (1939). *J. biol. Chem.* **129**, 491.
Querido, Lwoff & Lataste (1939). *C.R. Soc. Biol., Paris*, **130**, 1580.
Ritsert (1939). *Klin. Wschr.* **18**, 934.
Strafford & Parry Jones (1933). *Analyst*, **58**, 380.
Swaminathan (1938, 1). *Nature, Lond.*, **141**, 830.
—— (1938, 2). *Indian J. med. Res.* **26**, 427.
Vilter, Spies & Mathews (1938). *J. biol. Chem.* **125**, 85.
Vongerichten (1899). *Ber. dtsch. chem. Ges.* **32**, 2571.
Woolley, Strong, Madden & Elvehjem (1938). *J. biol. Chem.* **124**, 715.
Zincke (1904). *Liebigs Ann.* **330**, 361.

89. ESTIMATION OF NICOTINIC ACID IN ANIMAL TISSUES, BLOOD AND CERTAIN FOODSTUFFS

2. APPLICATIONS[1]

By E. KODICEK

From the Nutritional Laboratory, University of Cambridge and Medical Research Council

(*Received 29 March 1940*)

In the previous paper [Kodicek, 1940] the cyanogen-*p*-aminoacetophenone method for the estimation of nicotinic acid [Harris & Raymond, 1939, 1, 2] was tested on certain biological materials, and the individual steps of the reaction were examined in detail in order to ascertain the most accurate conditions for the application of the method. The procedure finally adopted has been used for determinations on animal tissues, blood and a number of foodstuffs and medicinal preparations. So far the only known biochemical action of nicotinic acid consists in hydrogen transfer in the phosphopyridine-nucleotide coenzyme systems. Therefore, for control tests the nicotinic acid content of preparations of cozymase was estimated and the concentration of cozymase was calculated from it and compared with the concentration found by the spectrographic method.[2] As yet it has not been found possible to distinguish between the coenzymes and free nicotinic acid, but an attempt was made to differentiate three separate fractions.

Working details

The method is in most respects almost identical with that described by Harris & Raymond [1939, 2] for urine, but, as success depends on close adherence to the minutiae of procedure, they are given here in full.

Reagents required. (1) CNBr solution: prepared fresh daily by adding ice-cold 10% aqueous solution of KCN drop by drop to ice-cold saturated Br water until it is just decolorized.

(2) Amino reagent: 5 g. *p*-aminoacetophenone and 30 ml. 32% HCl (sp.gr. 1·16), made up to 100 ml. with distilled water.

(3) 96% ethyl alcohol.

(4) 40% NaOH.

(5) 5% $NaHCO_3$.

(6) Standard solution of nicotinic acid: 100 μg. per ml. This solution is prepared weekly from a solution of 10 times the strength.

Process for animal tissues

(1) *Extraction, hydrolysis, alcohol precipitation and neutralization.* 0·5–2·0 g. of tissue are cut into small slices (only rarely was it necessary to grind the material) and placed with 5 ml. of distilled water plus 1 ml. of 40% NaOH in a small conical flask. The mixture is heated on a steam bath for 1 hr., a glass funnel

[1] Communicated to the Biochemical Society, 9 December 1939 [Kodicek, 1939].

[2] The spectrographic estimation was carried out by Dr D. E. Green, to whom I wish here to express my thanks.

being used to prevent excessive evaporation. The amide and other substances yielding nicotinic acid, such as phosphopyridine nucleotides, are converted by this hydrolysis into nicotinic acid. The mixture is cooled, and 40 ml. of 96% ethyl alcohol are added. The precipitate is centrifuged. The clear solution is neutralized accurately to *p*H 6·5 with conc. HCl in presence of 1–2 ml. of 5% $NaHCO_3$ to adjust the *p*H precisely, bromothymol blue being used as external indicator. The solution is made up with ethyl alcohol to a volume of 50 ml.

Notes. (*a*) The solution is sometimes slightly turbid, but clears when water or CNBr is added. If the natural colour of the material is too intense, a dilution to 100 ml. or more with ethyl alcohol is recommended.

(*b*) The amount of tissue taken for estimation should contain about 50 μg. of nicotinic acid.

(*c*) After neutralization the solution can be left overnight in the refrigerator without loss of nicotinic acid.

Example: Liver, estimated immediately ... 126 μg./g.
Liver, after 24 hr. ... ... 129 μg./g.

(2) *Opening of the pyridine ring and development of colour.* Four graduated 15 ml. stoppered flasks are taken (*X*, *A*, *B* and *C*). To flasks *B* and *C*, 0·2 and 0·4 ml. respectively are added of the standard solution of 10 mg./100 ml. of nicotinic acid (=20 and 40 μg. of nicotinic acid). 10 ml. of the prepared extract are run into each of the 4 flasks. Sample *X* is kept as the blank. All 4 flasks are placed in a water bath at 70–80° for *ca.* 10 min. Then 2 ml. of freshly prepared CNBr reagent are added to flasks *A*, *B* and *C*, which are mixed well and left for 5 min. in the water bath. To the blank, *X*, 2 ml. of distilled water are added instead. Then the 4 flasks are cooled for 5 min. in cold water in a dark room. 0·4 ml. of the amino reagent (see above) is added to all 4 flasks which are then filled to the 15 ml. mark with 96% ethyl alcohol. The contents are mixed and allowed to stand for 5 min. in a dark cupboard. Immediately afterwards, colorimetric measurements are taken with the Pulfrich photometer, using 3 cm. cells and filter S 47. Flask *X* is used as the blank, and samples *A*, *B* and *C* are examined for their contents of nicotinic acid.

Notes. (*a*) It is important not to expose the flasks to light after addition of the amino reagent, as the colour is sensitive to light.

(*b*) The dilution of the alcohol with water may cause a slight turbidity, measurable only by the sensitive photometer. The addition of 2 ml. of distilled water to the blank satisfactorily eliminates the possibility of error on this account. As the blank also contains the same amount of the amino reagent, another potential source of error is likewise eliminated. Similarly the colour of the extract itself does not interfere, as it is present also in the blank. The solution must be practically clear as the photometer acts also as a nephelometer.

(*c*) The amount of the amino reagent added must be accurately measured. During the course of the work it became clear, especially after the experiment illustrated in Fig. 1 of the previous paper, that a larger amount of amino reagent would be better, but during these series of estimations the original smaller amount of amino reagent was maintained. It is advisable to renew the *p*-aminoacetophenone weekly.

(*d*) The preparation of CNBr in ice-cold solutions was found to be important, as otherwise some formation of complex compounds probably takes place which may interfere [Kulikow & Krestowosdwigenskaja, 1930].

Calculations. From the 3 readings (say *A*, *B*, *C*) a graph can be constructed, which follows Beer's law and is a straight line—the colorimetric values being

taken as ordinates and the amounts of nicotinic acid added (viz. zero, 20, 40 μg.) as abscissae. This line when produced backwards cuts the horizontal axis at a point whose distance from the zero point represents the amount of nicotinic acid present in the "unknown" (sample *A*). By this procedure the amount of nicotinic acid can be estimated graphically, being dependent on the readings on the abscissae corresponding with the additions of 20 and 40 μg. of nicotinic acid. Fuller instructions will be found in the paper of Harris & Raymond [1939, 2].

It has proved of use to apply the following mathematical formula for calculating the content of nicotinic acid, thus eliminating the possibility of a small personal error in the graphical calculations.

$$\mu\text{g. of nicotinic acid per g. tissue} = \frac{A}{B+C-2A} \times \frac{6V}{n},$$

where *A*, *B* and *C* are the corresponding extinctions for the unknown (sample *A*), and for the unknown plus 20 and 40 μg. of nicotinic acid, respectively (samples *B* and *C*); V = the final total volume to which the extract was made up after neutralization, and n the number of grams of tissue taken for estimation.

Example: Extinction found for $A = 0{\cdot}21$, for $B = 0{\cdot}63$, for $C = 1{\cdot}05$; $n = 1$ (since 1 g. of fresh weight of tissue, pancreas, was taken for the estimation); $V = 50$ (neutralized extract made up to 50 ml.).

$$\text{Nicotinic acid} = \frac{0{\cdot}21}{0{\cdot}63 + 1{\cdot}05 - 0{\cdot}42} \times \frac{6 \times 50}{1} = 50\ \mu\text{g. per g. fresh tissue.}$$

The formula was calculated upon the basis of the observation that the higher colour values have a greater relative error than the lower, so that the mean of the extinction values *B* and *C* gives a fairer assessment of the true result. This mean value represents an extinction equal to 30 μg. of nicotinic acid.

Note. For routine work only 2 readings need be used, since the comparison with the standard reference curve gives another valuable check on the result. As a general rule, however, when working with unknown materials, it is advisable to adhere to the original method (i.e. 3 readings).

Results

(*a*) *Animal tissues and some medicinal preparations.* The results for various animal tissues are given in Table 1. The figures seem to run parallel with the reputed biological values. Liver and adrenals have the highest amount, then follow kidney, heart, muscle and other organs. It may be noted that the eye-lens has a fair amount of nicotinic acid and in human cataract no drop to zero was found as may happen with ascorbic acid [Müller, 1935; Hradecka *et al.* 1937; and others]. Salmon was very rich in nicotinic acid, which accords with previous biological findings [Goldberger & Wheeler, 1928].

The most potent source of nicotinic acid, however, was the proprietary liver extract Eli Lilly "343", which contained 1090 μg. per g. of powder, equivalent to 52 μg. of nicotinic acid per g. fresh weight of liver. Yeastrel and marmite are very rich. Baker's and brewer's yeasts have high concentrations of nicotinic acid allowing for the high content of water.

(*b*) *Dairy products (milk and eggs) and human milk.* From the values given in Table 2 it will be seen that a surprising finding is the very low content of nicotinic acid in milk, which varied from less than 1 to 5 μg. per ml. The estimations were made during the months of November and December. Since, owing to the sensitivity of the method, even less than 1 μg. can be detected,

Table 1. *Concentrations of nicotinic acid in animal tissues and medicinal preparations*

Material	Nicotinic acid, μg. per g. fresh wt. of tissue*	Reputed biological (pellagra-preventing) value
Liver (ox)	170	Good
Liver (sheep)	200	Good
Adrenals (sheep)	135	—
Heart (sheep)	60	—
Muscle, skeletal (ox)	43	Good
Kidney (sheep)	75	—
Pancreas (sheep)	40	—
Spleen (ox)	53	—
Lung (ox)	43	—
Brain (ox)	30	—
Eye-lens (ox)	50	—
Eye-lens, human, cataract	100	—
Fish:		
Salmon	84	Good
Herring	40	—
Cod	30	—
Roe, herring	21	—
Roe, turbot	23	—
Medicinal preparations:		
Liver extract, Eli Lilly "343"	1090	Very good
Yeastrel	400	—
Marmite	640	—
Yeast, baker's (moisture 69%)	74	Good
Yeast, brewer's (moisture 78%)	91	Good

* Results have been calculated as mean values, from at least three estimations.

Table 2. *Nicotinic acid in dairy products, milk and eggs, and human milk*

Material	Nicotinic acid μg. per ml. or g. fresh wt. respectively	Biological (pellagra-preventing value)
Egg-white	<0·5	None*
Egg-yolk	10	
Milk,† fresh, Nov.–Dec.	<1–5	Fair [Sebrell, 1934; Wheeler & Sebrell, 1933]
8 samples, av.	3	None*
Milk, dried	25	
Human milk:†		
Mrs E.	<1	
Mrs R.	<1	
Mrs M.	<1	

* Harris & Kodicek [1940] found egg-white and fresh milk inactive in blacktongue when given in curative tests in doses as high as 90 g. and 120 ml. per kg. wt. of dog, respectively.

† 2, 4 and 8 ml. of milk were used for the estimations; hydrolysis with 4, 8 and 10% NaOH yielded the same low results.

there seems no doubt that the amount of nicotinic acid in milk is indeed negligible, and especially is this so for human milk. The higher concentration of nicotinic acid in dried milk corresponds well with the amount found in fresh milk calculating on the wet basis. Recovery of added nicotinic acid was 105% [cf. previous paper: Kodicek, 1940].

Egg-white was found to be negative, whereas the yolk contained 10 μg. per g. fresh weight. The biological values for milk given by Wheeler & Sebrell [1933] and Sebrell [1934][1] do not agree with these chemical findings, but in our own

[1] See Discussion, below.

experiments [Harris & Kodicek, 1940] we were able to confirm the negative result. Even 120 ml. of fresh milk per kg. of body weight daily failed to cure blacktongue in dogs. Egg-white likewise proved to be deficient when tested on dogs in curative or preventive doses of 100 g. daily [Harris & Kodicek, 1940].

(*c*) *Blood.* A special modification was worked out as follows: 5 ml. of oxalated blood are hydrolysed with 0·5 ml. of 40% NaOH for 1 hr. on a boiling water bath. After cooling, 50 ml. of acetone are added, the mixture is centrifuged and the clear solution neutralized. The contents are made up to the 50 ml. mark with

Table 3. *Estimation of nicotinic acid in blood*

Description	A Estimated in acetone solution.* Nicotinic acid μg. per ml.	B Acetone evaporated off, estimation carried out in ethyl alcohol.† Nicotinic acid μg. per ml.
Horse blood:		
Whole blood, oxalated	4·7	4·8
Red cells	14·0‡	—
Serum	0·0	0·0
Plasma	0·0	0·0
Sheep blood:		
Whole blood, oxalated	4·7	4·5

* A = 5 ml. of oxalated blood hydrolysed with aqueous NaOH and estimated in acetone solution in usual manner.

† B = 5 ml. of oxalated blood hydrolysed with aqueous NaOH, 50 ml., of acetone added, solution centrifuged till clear, acetone evaporated off *in vacuo*, residue dissolved in 96% ethyl alcohol, made up to 50 ml. and estimated.

‡ The value for whole blood calculated from the haematocrit reading gives 4·5 μg. per ml. of whole blood, in good agreement with the direct experimental estimation.

Table 4. *Different fractions of nicotinic acid in blood (horse)*

Description	Nicotinic acid μg. per ml. whole blood
"Fraction 1". Unhydrolysed alcohol-soluble fraction (free nicotinic acid plus one-fifth of amide)	0·0
"Fraction 2". Hydrolysed alcohol-soluble fraction (nicotinic derivatives soluble in alcohol, which are converted into nicotinic acid upon hydrolysis)	0·0
"Fraction 3". Alcohol-insoluble fraction hydrolysed (residuum consisting of derivatives insoluble in ethyl alcohol, 96%)	5·0
"Fraction 4". Unhydrolysed acetone-soluble fraction	0·0
"Fraction 5". Hydrolysed acetone-soluble fraction	0·5
"Fraction 6". Acetone-insoluble fraction hydrolysed	4·0

"Fraction 1" was obtained by the following treatment: 5 ml. of whole oxalated horse blood were extracted with 40 ml. of 96% ethyl alcohol for 30 min. at 30° and vigorously stirred. The mixture was centrifuged, the clear solution made up with ethyl alcohol to 100 ml. and estimated without hydrolysis.

"Fraction 2". The same as "Fraction 1", but 50 ml. of the final solution taken and hydrolysed with 5 ml. of 40% NaOH using a reflux cooler, then the nicotinic acid estimated as usual.

"Fraction 3". The residuum obtained after the centrifuging of "Fraction 1" was hydrolysed with 5 ml. of distilled water and 0·5 ml. of 40% NaOH.

"Fractions 4, 5 and 6" are similar to "Fractions 1, 2 and 3", respectively, but acetone was used for extraction instead of ethyl alcohol.

96% ethyl alcohol and the estimation is carried out as described for animal tissues. Table 3 shows the results and the effect of different modifications in procedure.

No nicotinic acid was found in serum and plasma, the whole amount being in the red blood cells. As the simpler method with acetone described under A gives the same results as estimations in ethyl alcohol, the former was adopted as a routine.[1] Only a few examples of the estimations on blood are given in this paper, and more extensive results will be presented later.

In the work summarized in Table 4 an attempt was made to differentiate various forms of nicotinic acid derivatives present in blood. The methods used were the same as those adopted for animal tissues (see Table 8 in this paper, where more details will be given). From these findings it may be concluded that the whole of the nicotinic acid, which is present only in the red blood cells, is bound to substances which are insoluble in 96% ethyl alcohol and probably also in acetone. At present it is not certain whether the whole of the nicotinic acid can be attributed to phosphopyridine nucleotides or whether there are also other substances containing the nicotinic acid grouping.

The results in Table 4 are in good agreement with values obtained by quite a different procedure and recorded in Table 3, the total nicotinic acid being in the former 5·0 and 4·5 μg. of nicotinic acid per ml. of whole blood, respectively, and in the latter 4·7 and 4·8 μg. per ml., respectively.

(*d*) *Cereals and vegetables.* Table 5 shows the results of tests on cereals and vegetables. As has already been stated in the previous paper, certain difficulties appeared in the interpretation of the results for these substances. No such trouble was encountered with animal tissues, from which it was found that all the nicotinic acid could be readily extracted with boiling water. This suggests that in animal tissues all the nicotinic acid is present in the free state, or bound to substances soluble in water (such as phosphopyridine nucleotides) or very easily split off by boiling water. This question will be dealt with later. But in the case of some cereals it was found that only a small part of the chromogen ("apparent" nicotinic acid) passed into solution upon extraction with water at 100°. As it seemed certain that this behaviour was not due to imperfect extraction, it was necessary to assume that some unknown substance giving the cyanogen-*p*-aminoacetophenone reaction was more strongly bound and liberated only after hydrolysis with NaOH. This difference between cereals and animal tissues is of some importance, and it was not at first certain which of the two methods of extraction—by hydrolysis (extractions *a* and *b*) or by boiling water (extraction *c*)—gave the true content of nicotinic acid which would correspond with the biological test. Such discrepancies between the reputed biological values of these cereal foodstuffs and the chemical values as obtained after the more drastic method of extraction supported the view that some unspecific chromogen was being set free which was not in fact active as a pellagra-preventing substance.[2] During the course of the present investigations Harris & Kodicek [1940] were able to test this theory. In experiments on dogs with blacktongue, a specimen of yellow maize flour (sample *C*), which showed chemically an unusually high content of the "apparent" nicotinic acid, 27 μg. per g. material, was tested. It was found in fact that this sample was just as deficient as the others examined at the same time, 4 dogs developing blacktongue in the usual period of about

[1] With blood, acetone was found to be preferable to alcohol since a clear solution free from pigments was more readily obtained.

[2] The possibility of such an unspecific reaction was discussed in greater detail in the previous paper.

Table 5. *Estimation of chromogens ("pyridine-like substances") in cereals*

Method of extraction ... (see below)	Chromogens calculated as μg. of nicotinic acid per g. of material				Biological pellagra-preventing values [Sebrell, 1934]
	a	*b*	*c*	*d*	
Cereals:					
White flour	5	8	—	—	—
	12	—	—	—	—
Hovis flour	26	—	—	—	—
Whole wheat	33	—	—	—	Slight
Wheat germ	39	38	27	17	Good
White bread (moisture, 31%)	<5	—	—	—	—
Brown bread (moisture, 34%)	25	28	12	13	—
	33	—	—	—	—
Rice powder	16	—	—	12	—
	27	26	17	5	—
White maize meal	13	—	—	—	Deficient*
Yellow maize meal:					
Sample A	10	—	—	—	Deficient*
Sample B	18	33	—	—	—
Sample C	12	27	6	—	Deficient†
Sample D	41	45	8	37	—
Pea meal	49	—	—	—	—
	25	20	18	5	—
Caseinogen ("Glaxo", A/E)	<5	—	—	—	—
Sugar	<2	—	—	—	—
Starch, rice, raw	<5	—	—	—	—
Goldberger diet:					
I	10	—	—	—	Deficient*
II	17	26	6	20	Deficient†
Vegetables:					
Potatoes	20	—	—	—	None
Spinach	17	—	—	—	Fair
Cabbage	3	—	—	—	Fair
Carrots	<5	—	—	—	Slight
Tomatoes	<5	—	—	—	Good
Apples	<5	—	—	—	None

* Harris & Kodicek [1940] have used these cereals successfully in the production of black-tongue. Goldberger diet I contained: white maize meal 4000, pea meal 500, extracted casein A/E (Glaxo) 600, salts 130, cottonseed oil 300. If the content of nicotinic acid is calculated for this diet from the individual constituents, it is found to be 13 μg. per g., which is in good agreement with the figure obtained by actual determinations on the whole diet itself.

† Goldberger diet II contained the same amount of yellow maize meal (sample C) instead of white maize meal.

Extractions. The different methods of extraction have been more fully discussed in the previous paper, Table 1 [Kodicek, 1940].

a = Extracted during hydrolysis with aqueous NaOH = total chromogens, active plus inactive.

b = Extracted during hydrolysis with alcoholic NaOH = total chromogens, active and inactive.

c = Extracted with water at 100° for 1 hr., centrifuged and solution hydrolysed = active nicotinic acid (?).

d = Residuum left after water extraction *c*, extracted during hydrolysis with alcoholic NaOH.

10–30 days. The water extract of this sample yielded very low chemical values—6 μg. per g.—which would agree with the negative biological result. Moreover, the solubility in boiling water runs parallel with the behaviour of the active nicotinic acid present in animal tissues from which it appears to be completely extracted by water. It is therefore probable that extraction with boiling water is the process which shows the true content of "active" nicotinic acid.

In Table 5 some results are given in column *c* obtained with this procedure. There is, however, no need to assume that the substance so extracted from

cereals by boiling water is necessarily identical with the active pellagra-preventing substance until experiments, which are now in progress, have proved this suggestion to be true or otherwise.

No explanation has yet been found why, after hydrolysis in water, lower figures are sometimes found than after hydrolysis in ethyl alcohol.

Stability of nicotinic acid

It is known that nicotinic acid is very stable to heat and many kinds of chemical treatment. Table 6 shows that the storage of foodstuffs for as long as 3 years did not lower their content of nicotinic acid. Also urine stored for 1 month showed no appreciable loss of nicotinic acid.

Table 6. *Stability of nicotinic acid*

Material	Nicotinic acid μg. per g.
Yeastrel stored:	
(*a*) Since Nov. 1936	380
(*b*) Since Sept. 1937	440
(*c*) Since June 1938	360
(*d*) Since Aug. 1939	320
Urine, human, preserved with sulphur-free toluene in refrigerator:	
1st day	12
8th day	12
18th day	10
25th day	14

Table 7. *Recovery of nicotinic acid from cozymase*

Description	Amount of cozymase taken, expressed as nicotinic acid* μg.	Amount of nicotinic acid found μg.	Recovery of nicotinic acid %
Cozymase (Euler), 90% purity:			
(*a*) Without hydrolysing, "free nicotinic acid"	17	0	0
(*b*) Hydrolysed in 5 ml. with 2 ml. NaOH, 20%	17	15	88
Cozymase (Green), sample A, 11·3% purity:			
(*a*) Without hydrolysing, "free nicotinic acid"	45	0	0
(*b*) Hydrolysed in 5 ml.			
(1) With 1 ml. NaOH, 20%	45	37	82
(2) With 2 ml. NaOH, 20%	45	40	89
Cozymase (Green), sample B, 52% purity:			
(*a*) Without hydrolysing, "free nicotinic acid"	58	0	0
(*b*) Hydrolysed in 5 ml. with 2 ml. NaOH, 20%	58	56	97

* The amount of nicotinic acid was calculated from the concentration of pure cozymase determined spectrographically by Dr D. E. Green after enzymic reduction to dihydrocozymase. Molecular weight of cozymase was taken as 681; that of nicotinamide is 122 [Lutwak-Mann, 1939].

Recovery of nicotinic acid from cozymase

Since the main if not the only source of nicotinic acid in animal tissues is probably the phosphopyridine nucleotides, the amount of nicotinic acid in different preparations of cozymase was estimated (Table 7) and compared with the calculated amount, the degree of purity of these different enzyme specimens

being determined spectrographically by Dr D. E. Green. No so-called "free nicotinic acid" was found in untreated preparations of cozymase. The recovery of nicotinic acid from preparations of cozymase was calculated to be from 88 to 97%. The linkage of nicotinic acid in cozymase is very weak, and after heating for 60 min. on a steam bath nicotinic acid already appeared in the free state in a concentration of 13%. As it is probable that it was the amide in solution which was being estimated, a five-times higher value ought to be calculated, the nicotinamide when unhydrolysed giving only 20% of the colour of nicotinic acid itself (see section on the recovery of nicotinamide in previous paper). It may therefore be concluded that as much as *ca.* 65% of nicotinamide would be liberated simply by boiling for 60 min.

Differentiation of various fractions

Using three different methods of treatment three different sets of results have been obtained for animal tissues, and this may indicate that nicotinic acid in animal tissues can be divided into three or more different fractions. This suggestion is, however, at present nothing more than provisional. Table 8 shows

Table 8. *Different fractions of nicotinic acid in animal tissues and some other substances*

	"Fraction 1" Unhydrolysed alcohol-soluble fraction "Free nicotinic acid", μg. per g. fresh wt.	"Fraction 2" Hydrolysed alcohol-soluble fraction Nicotinic acid μg. per g. fresh wt.	"Fraction 3" Total nicotinic acid μg. per g. fresh wt.
Animal tissues:			
Liver (ox)	20	90	170
Liver (sheep)	25	120	200
Adrenals (sheep)	24	66	135
Heart (sheep)	15	20	60
Muscle skeletal (ox)	7	11	43
Kidney (sheep)	25	80	75
Pancreas (sheep)	10	30	40
Brain (ox)	8	10	30
Spleen (ox)	0	63	53
Blood (horse)	0	0	4·7
Other materials:			
Liver powder, Eli Lilly "343"	250	1030	1090
Do.: calculated per g. fresh wt. of liver	12	49	52
Yeastrel	380	380	400
Marmite	600	580	640
Baker's yeast (moisture 69%)	10	25	74
Brewer's yeast (moisture 78%)	14	60	91

"Fraction 1." Material first extracted with 96% ethyl alcohol by grinding with sand or stirred for 2 hr., and without subsequent hydrolysis = "unhydrolysed alcohol-soluble fraction", representing free nicotinic acid plus 20% of free amide.

"Fraction 2." Same as "Fraction 1" but subsequently hydrolysed = "hydrolysed alcohol-soluble fraction".

"Fraction 3." Original material or aqueous extract first hydrolysed as a whole = "total nicotinic acid".

the results expressed as different fractions. "Fraction 3" is the total nicotinic acid present in animal tissues; "Fraction 2" is the hydrolysed alcohol-soluble fraction, which consists of substances soluble in 96% ethyl alcohol. This fraction is partly the nicotinic acid and amide in the free state and partly alcohol-soluble

'bound nicotinic acid" which only gives the cyanogen-*p*-aminoacetophenone reaction after hydrolysis. The latter portion can easily be calculated, knowing the concentration of free nicotinic acid and roughly that of the amide, estimated in "Fraction 1". In "Fraction 1" the unhydrolysed alcohol-soluble part is estimated, which seems to consist of free nicotinic acid plus one-fifth of the free amide, since unhydrolysed amide yields only 20% of the maximum colour.

The differences between the total nicotinic acid and the fraction obtained on extraction with ethyl alcohol before hydrolysis (hydrolysed alcohol-soluble fraction) are not due to imperfect extraction of the latter. No difference was found whether the alcohol extraction was performed simply by grinding the tissue with sand in ethyl alcohol or by stirring for 2 hr. at 50°. Nor is the variation due to the difference in *p*H. It is true that the "alcoholic fraction" is extracted at a neutral reaction and that the "total nicotinic acid" fraction is treated with ethyl alcohol at a very strong alkaline reaction after hydrolysis: control tests showed, however, that neutralization of the hydrolysed total nicotinic acid extract "Fraction 3" before addition of ethyl alcohol did not alter the results.[1] The different figures for the various fractions must therefore be due either to incomplete precipitation of certain nicotinic acid derivatives or else to selective precipitation of certain fractions. How the phosphopyridine nucleotides will behave in this direction has not yet been examined. Liver, adrenals, heart, brain and muscle showed notable differences between the total amount of nicotinic acid and the hydrolysed alcohol-soluble fraction, indicating that in these tissues a considerable portion of the nicotinic acid was estimated in a form which is insoluble in ethyl alcohol. The possible effect of autolysis in causing such difference has still to be examined.

Yeastrel and marmite showed no differences as between the 3 fractions, indicating no doubt that during their preparation the nicotinic acid had been liberated, probably by autolysis. In Eli Lilly "343" liver powder only the total and hydrolysed alcohol-soluble fractions gave similar figures. The unhydrolysed fraction was only 25% of the total nicotinic acid. This may mean that in this preparation this small part only is present as free nicotinic acid, or that it is present entirely as amide, which gives a less intense colour if unhydrolysed. It is interesting to observe that this product, which is presumably prepared from alcoholic extracts of liver, contains nearly the same amount of nicotinic acid, calculated for fresh weight of liver, as the hydrolysed alcohol-soluble fraction of liver described in this paper (52 and 90 μg. per g. fresh liver, respectively).

The unhydrolysed alcohol-soluble fraction, the so-called "free nicotinic acid", is present only in traces in most tissues, and it is possible that it appears only on autolysis and that no free nicotinic acid is present as such in animal tissues. In horse blood all the nicotinic acid is present in substances which are precipitated by ethyl alcohol, so that both the hydrolysed and unhydrolysed alcohol-soluble fractions yield no nicotinic acid.

Discussion

The concentrations of nicotinic acid found in animal tissues agree fairly well with the reputed biological values. The high concentration in the eye-lens is interesting as nicotinic acid, being concerned with hydrogen transfer in phosphopyridine nucleotides, may play an important role in the respiration of the eye-

[1] Added nicotinic acid (100 μg.) could be recovered to the extent of 110 and 90% in "Fraction 2" of liver; in unhydrolysed alcohol-soluble fraction added nicotinic acid could be recovered to the extent of 100%.

lens as possibly also does ascorbic acid. The latter, unlike nicotinic acid, appears however to be diminished in cataract.

Whereas the results with animal tissues seem unequivocal, it was found that cereals contain some interfering substance or substances, possibly pyridine derivatives, which, though giving a positive colour reaction, are inactive in black-tongue. It seems likely that extraction with boiling water will prove to be more specific, but this is a matter for further experiments which are in progress. This procedure certainly gives significantly lower results for cereals and is a reliable procedure for animal tissues. At present no final conclusions can be given about cereals.

A surprising finding is the very low concentration of nicotinic acid in milk. Wheeler & Sebrell [1933] and Sebrell [1934] estimate the pellagra- and black-tongue-preventing value of milk as "fair". Amounts of 30 ml. per kg. weight of dog daily delayed the onset of blacktongue in dogs for a considerable length of time in their experiments. Milk is also recommended in nearly all text-books as being a good source of the pellagra-preventing factor for patients suffering from pellagra. However, dogs which we have kept on a blacktongue-producing diet [Harris & Kodicek, 1940] showed no improvement even after doses of 120 ml. of fresh milk per kg. body weight, nor in prophylactic tests did doses of about 30 ml./kg. delay the appearance of symptoms of blacktongue beyond the time observed for comparable deficient control animals (e.g. in *ca.* 2–4 weeks). This biological result therefore confirms the chemical determination. Again, human milk was found chemically to be deficient. This result raises questions about the metabolism of the growing organism. As infants need nicotinic acid for their coenzyme systems, it would be interesting to ascertain whether infants are born with a large store of nicotinic acid or have some means of synthesizing it.

In blood nicotinic acid was found only in the red cells, and in a form which is totally precipitated by ethyl alcohol and acetone. Whether the total nicotinic acid in blood is derived only from phosphopyridine nucleotides cannot at present be ascertained, but it seems highly probable.

The differentiation of various fractions is so far nothing more than provisional. It seems that in animal tissues free nicotinic acid appears very rapidly on autolysis, but that in living tissues it is not present, or else is present in only minute amounts. The rate of appearance of free nicotinic acid (or amide) would thus be an expression of the concentration of enzymes which are able to split the phosphopyridine nucleotides and other nicotinic derivatives. According to this point of view kidney, pancreas and spleen would be rich in such enzymes. More work is needed to differentiate the coenzymes from other possible sources of nicotinic acid in tissues, and investigations are in progress.

Summary

1. The cyanogen-*p*-aminoacetophenone method of Harris & Raymond [1939, 1, 2] was applied to the estimation of nicotinic acid in numerous animal tissues, medicinal preparations, blood, dairy products and cereals. The results were compared with the reputed biological pellagra-preventing values, as given by other authors or as found in new experiments in this institute.

2. Results for animal tissues run parallel with the biological values. The highest values were found for liver and adrenals. Salmon is rich in nicotinic acid. Eye-lens, normal and with cataract, contains a fair amount.

3. Egg-white was found to be deficient, both in chemical and biological tests. Milk was also found to be surprisingly low, and this was confirmed in experiments on canine blacktongue.

4. In horse and sheep bloods about 4·7 μg. of nicotinic acid per ml. were found, all of it in the red cells. The whole of the nicotinic acid seems to be bound to substances which are insoluble in excess of ethyl alcohol and in acetone.

5. Substances are present in cereals which give a non-specific colour reaction and complicate the estimation of the active nicotinic acid. It seems probable that extraction with water may be used to separate the active antiblacktongue principle from interfering substances.

6. Storage of certain food extracts over a period of years did not diminish their contents of nicotinic acid.

7. Cozymase was found to be quantitatively hydrolysed and recovered (viz. 88–97 %).

8. Nicotinic acid is present in various forms of combination and an attempt was made at a provisional differentiation of the different fractions. Certain tissues, namely liver, adrenals, heart and skeletal muscle, appeared to contain various fractions, differentiated by their insolubility in ethyl alcohol.

9. It appears that little or no free nicotinic acid is present in living animal tissues but that on autolysis it is rapidly set free from coenzymes or other combined forms.

I am glad to be able to express my profound gratitude to Dr L. J. Harris for his great help and invaluable advice. I am indebted to Sir Charles Martin and Dr D. E. Green for providing me with samples of cozymase, and to the latter also for the spectrographic estimation of cozymase preparations; Messrs Eli Lilly and Co., Ltd., have generously provided supplies of their liver extract "343". I also wish to thank the Society for the Protection of Science and Learning for a grant and support during this work.

REFERENCES

Goldberger & Tanner (1924). *Publ. Hlth Rep., Wash.*, **39**, 3.
—— —— (1925). *Publ. Hlth Rep., Wash.*, **40**, 2.
—— & Wheeler (1928). *Publ. Hlth Rep., Wash.*, **43**, 23.
Harris & Kodicek (1940). In the Press.
—— & Raymond (1939, 1). *Chem. Ind.* **58**, 652.
—— —— (1939, 2). *Biochem. J.* **33**, 2037.
Hradecka, Joachim & Kodicek (1937). *Ceskoslovenska Ophthalmol.* **3**, 1.
Kodicek (1939). *Chem. Ind.* **58**, 1088.
—— (1940). *Biochem. J.* **34**, 712.
Kulikow & Krestowosdwigenskaja (1930). *Z. anal. Chem.* **19**, 452.
Lutwak-Mann (1939). *Biol. Rev.* **14**, 399.
Müller (1935). *Arch. Augenheilk.* **109**, 304.
Sebrell (1934). *Publ. Hlth Rep., Wash.*, **49**, 754.
Wheeler & Sebrell (1933). *Nat. Inst. Hlth Bull.* **1**, 162.

90. THE VITAMIN A, CAROTENE AND XANTHOPHYLL CONTENT OF THE YOLK OF HENS' EGGS

By B. SJOLLEMA and W. F. DONATH

From the Institute for the Studying of Animal Nutrition, "de Schothorst", Hoogland

(*Received 21 March 1940*)

In the course of experiments with chickens carried on in this institute on the importance of vitamin A, carotene and xanthophyll in the diet of hens for their egg production, health, the hatchability of their eggs, the vitamin A in their liver and the viability of the chicks produced from these eggs, a large number of yolks were examined for their content of these three substances.

The results of these studies are the subject of the present paper.

Experimental

There were about 300 hens at our disposal—White Leghorns in their first production period. About 180 of these, i.e. groups A–H, were housed indoors in all-metal individual cages. This made it possible to keep a check on the food taken and the eggs laid by each hen. About 120 hens, i.e. groups V–VII, were housed in pens of which the exits were kept closed. None of the animals were given a chance to eat fresh grass or any other green food.

The diet given to the various birds differed only in respect of the constituents rich in the three above-named substances. Vitamin A was administered as dohyfral oil, which contains shark's oil and is also a source of vitamin D_3. Yellow maize and dehydrated alfalfa were used as sources of carotene. One group, namely group G, was given food in which all these constituents were lacking. In the groups without dohyfral in the diet vitamin D_3 was given instead. The other components of the hens' diet were very poor in the three substances under consideration.

The diet consisted of a mash, which was given dry, and grain, a mixture of unground maize, barley, wheat and oats (25% of each) and in addition $2\frac{1}{2}$% of sunflower seeds.

The composition of the mash as originally given was:

	%
Maize	25
Wheat middlings	20
Barley flour	15
Oatmeal	10
Soya bean flour	10
Meat and bonemeal	10
Fish meal	6
Dried yeast	1
Mineral mixture	2·5
Dohyfral oil	0·1

This food was given to all the animals before experimentation began (8 December 1938). It was fed to groups C, D and VI throughout the whole experiment. The dohyfral oil contained about 1800 I.U. of vitamin A per g.

The pullets consumed about 110 g. of food daily. The quantity of the mash taken was as a rule not quite equal to the quantity of grain eaten. Most of the groups took rather more of the mash than of the grain. Nevertheless in the following we shall assume that the amounts were the same.

Where alfalfa was added to the mash, the amount of wheat middlings was correspondingly reduced.

The experiment took 9 months, from 2 February until 8 November 1939.

The following table shows the sources of carotene and vitamin A in the food of the different groups.

Group	A	B	C	D	E	F	G	H	V	VI	VII
Yellow maize 25%	+	+	+	+	+	−	−	−	+	+	+
Alfalfa	+(5%)	+(5%)	−	−	−	(+5%)	−	+(7·5%)	+(5%)	−	+(7·5%)
Dohyfral 0·05%	+	−	+	+	−	−	−	−	−	+	−

Groups A and C were formed on 2 February; B, D, V, VI and VII on 14 February; E and F on 17 March; and G and H on 4 April 1939.

In diets (groups F, G and H) from which yellow maize was omitted this was replaced by an equal quantity of white maize in the mash as well as in the grain mixture.

Vitamin A, carotene and xanthophyll were determined after sponification as recommended by Wolff [1938–9], by extraction with peroxide-free ether, after which Carr and Price's reaction was applied to determine the amount of vitamin A using the Pulfrich Stufenphotometer. To determine not only the vitamin A content but also the carotenoids the method had to be somewhat modified and amplified. The method described by Baumann *et al.* [1939] did not quite satisfy us in that it seemed rather complicated.

After discussing the matter with Mr Emmerie, whom we are glad to take the opportunity of thanking for the help he gave us and the interest he showed in our research, we decided to use the following method.

The analytical method

1. *Yolks.* After being weighed, the yolk is saponified with 125 ml. 10% alcoholic KOH for 1½–2 hr. in a water bath using a reflux condenser. An equal volume of water is then added and the mixture, after being allowed to cool for 1 hr. in a refrigerator, is shaken three times with ether free from peroxide (75, 60 and 50 ml. respectively) in a 500 ml. separating funnel. The combined ether extracts are subsequently washed with water until they are entirely free from alcohol (generally three careful washings with 100 ml. H_2O are sufficient; the formation of emulsion may be prevented by means of a little alcohol). After being put to dry all night over anhydrous sodium sulphate, the ether is divided into two parts. The first part is evaporated in a stream of CO_2 and the residue dissolved in about 5–10 ml. $CHCl_3$, sometimes more, according to the amount of vitamin A expected to be present. With the antimony trichloride reagent the total blue coloration derived from the vitamin A, carotene and xanthophyll is determined using the Pulfrich Stufenphotometer, with filter S 61 and a 0·5 cm. cell. The Lovibond blue units in the half-yolk may then easily be read by the aid of the curve.

The second part of the ether extract is also evaporated in an atmosphere of CO_2 (in a vacuum) and the residue dissolved in 75 ml. light petroleum (B.P. 60–80°) and then shaken in a separating funnel with 85% alcohol until the latter has become totally colourless. In the case of not too strongly coloured yolks extraction with 75, 50, 50 and 40 ml. respectively is sufficient. The light petroleum fraction then contains the carotene while the xanthophyll is in the

combined alcoholic fractions.[1] The carotene solution is then washed twice with 50 ml. H_2O and left to dry all night in the refrigerator over anhydrous sodium sulphate. After evaporation (in a stream of CO_2 and in a vacuum) on the water bath, the residue is dissolved in light petroleum (B.P. 60–80°). The amount of light petroleum needed depends on the carotene content of the yolks. For light-coloured yolks we used 5–10 ml.; for strongly coloured ones up to 25 ml. By the use of filter S. 47 of the Stufenphotometer using a 0·5 cm. cell, and a curve constructed for pure β-carotene, the carotene content of the half-yolk may be determined.

The alcoholic solution of xanthophyll derived either from the whole yolk in the case of light-coloured yolks or else from a part, is diluted with at least five times its own volume of H_2O; then 50 ml. light petroleum are added, after which the mixture is well shaken and the water removed. Then the xanthophyll solution is again washed twice with 100 ml. H_2O and dried overnight in the refrigerator with anhydrous sodium sulphate. Like the carotene the xanthophyll derived from the half-yolk is then, after being evaporated in the water bath in a stream of CO_2 in a vacuum, dissolved in light petroleum, the amount of which varies from 5 to 25 ml. according to the quantity of xanthophyll present. By the aid of the Stufenphotometer and using a 0·5 cm. cell and filter S. 47, the calculation is made by means of a curve obtained for pure xanthophyll.

Since not only vitamin A but also carotene and xanthophyll give a blue coloration with antimony trichloride, it still remains to calculate the Lovibond blue units of the sum of the amounts of carotene and xanthophyll. This is done by means of a fourth curve which gives the blue value in photometer units. The value found on this curve can be converted into Lovibond units by the aid of the first curve. Subtraction of this number of Lovibond units from the number of Lovibond units previously found, gives the amount of vitamin A expressed in Lovibond units in the half-yolk. Multiplying this figure by 6·4 gives the same expressed as International Units. To find the vitamin A, carotene and xanthophyll contents of the whole yolk the above figures must be multiplied by 2.

When determining the vitamin A contents of the cod liver oil and dohyfral oil used, we kept strictly to the results given by Wolff [1938–9] for cod liver oil and assumed that the very light yellow colour of some extracts might be neglected.

2. *Poultry feed.* The method we followed in examining poultry feed was, in general, also the one indicated by Wolff [1938–9] and consisted of saponification and extraction with light petroleum and the subsequent determination of carotenoids. In analysing foodstuffs in which vitamin A might be expected as a result of adding cod liver oil, dohyfral oil or fish meal, we followed the same technique as in determining vitamin A, carotene and xanthophyll in yolks of eggs. This was permissible because the components of the feed could not be expected to contain any carotenoids (such as lycopene) other than carotene and xanthophyll, at least in more than traces.

From 5 to 15 g. of the feed to be examined were used, according to the amount of carotene that we expected to find, the sample having previously been ground so that it passed a 0·45 mm. sieve.

5 g. of finely ground maize for instance were saponified for 2 hr. with 75 ml. alcoholic 15% KOH. After being cooled and diluted with an equal volume of water the liquid is put into a separating funnel and extracted with light petroleum (B.P. 60–80°) until no more yellow substance is transferred to the petroleum

[1] We call this fraction the xanthophyll fraction without wishing to imply that it contains only this carotenoid.

(generally four extractions with 60, 50, 40 and 40 ml. light petroleum are sufficient). The collected extractions are then washed with an equal volume of water. After this they are washed once with 50 ml. 0·5*N* KOH and then with H_2O until the petroleum is entirely free of alkali (generally three washings are sufficient). Then the carotene and xanthophyll are separated with 85% alcohol, as described in the case of yolks. The further treatment corresponds to the one described above. For the calculation curves 2 and 3 are used.

In the case of the poultry feed containing carotenoids as well as vitamin A the light petroleum extract was divided into two equal parts and treated as described for yolks, or, if little of the active substance was to be expected in the feed, we made a separate analysis to determine the total vitamin A and carotenoids and a second one for the carotenoids alone, from which two analyses we were able to calculate the vitamin A content.

From the above it is clear that we did not use the biological method for determining the vitamin A and carotene contents of the yolks and feeds. At the end of the present paper we discuss the results of experiments in which the biological method was used, which in our opinion gave results concordant with ours. As far as we can see the physico-chemical method here employed gives results which, in the case of the materials we are dealing with, agree with those obtained biologically.

Results

I. *Vitamin A, carotene and xanthophyll contents of yolks of eggs laid by hens fed on a diet poor in these substances, and the effects of supplementing the diet*

How low the content of these substances may be in the yolk of an egg is shown by examination of the yolks of group G, whose diet contained no constituents rich in carotene.

The results obtained from a study of this group also show what effect may be produced on the yolk in respect of its content of these substances by supplementing the diet of hens with suitable additions.

The mash fed to these hens during rather more than 2 months contained 1·5 μg. carotene plus xanthophyll per g. Examination of the fish meal revealed that it contained about 25 I.U. of vitamin A per day's ration. The grain only contained 0·25 μg. carotene per g.

The following table gives the contents of the yolks of eggs of hens from group G which were given from 14 June onwards the supplemented diet containing vitamin A or carotene. The figures found to the right of the line refer to eggs laid after the supplementary feeding began.

The table given below presents the vitamin A in I.U., the carotene and xanthophyll content as μg. per yolk.

From this table it is evident how low the content of the yolks had become before 14 June and how much it rose even 2 weeks after the hens were given either alfalfa, cod liver oil (daily 4 ml. containing 740 I.U. vitamin A) or dohyfral oil (daily 0·42 ml. containing 739 I.U. vitamin A) or dehydrated alfalfa to the amount of 15% of the mash, so that every day the hens consumed about 8 g. of alfalfa containing about 1160 μg. carotene.[1]

The average vitamin A content of the eggs of all the hens of group G was 58·5 in the first half of June; in May, 78 and in April, 137 I.U.[2]

[1] Before July the 1938 crop of lucerne was used which contained 104 μg. carotene. The alfalfa used afterwards contained 141 μg. per g.

[2] In calculating this average the eggs of one hen (No. 45) which differed markedly from all the others, were not taken into account.

I. Effect of dohyfral oil

Hen No. 29: Date ...	5. vi.	20. vi.	26. vi.	29. vi.–7. ix Average of 4 yolks
Vitamin A	52·2	99·9	173·3	185·3
Carotene	6·8	6·1	6·8	12·0
Xanthophyll	42·2	45·8	36·4	34·2

Hen No. 38: Date ...	16. v.	26. v.	16. vi.	19. vi.	20. vi.	22. vi.	28. vi.–7. ix. Average of 3 yolks
Vitamin A	89·6	103·3	70·7	77·3	128·0	171·5	177·6
Carotene	3·0	4·4	6·7	6·8	4·0	3·4	8·6
Xanthophyll	35·8	29·0	18·9	31·5	39·5	30·2	33·5

Hen No. 39: Date ...	16. v.	25. v.	6. vi.	18. vi.	20. vi.	21. vi.	22. vi.	27. vi.–4. ix. Average of 3 yolks
Vitamin A	113·0	90·2	68·8	61·1	86·9	114·5	159·4	174·0
Carotene	2·0	9·4	9·2	5·7	5·8	6·0	7·2	7·7
Xanthophyll	40·2	20·4	22·8	40·4	38·4	37·6	38·0	40·2

II. Effect of cod liver oil

Hen No. 12: Date ...	23. iv.	5. v.	9. v.	26. v.	28. v.	30. vi.	1. vii.–6. ix. Average of 3 yolks
Vitamin A	105·0	51·2	32·0	46·9	18·5	108·8	145·5
Carotene	7·6	8·6	3·8	7·8	6·9	5·2	6·9
Xanthophyll	59·0	33·6	17·6	22·4	27·4	36·0	35·5

Hen No. 17: Date ...	31. v.	17. vi.	3. vii.	15. vii. Average of 2 yolks	15. vii.–17. vii.
Vitamin A	72·4	59·5	131·8	162·8	
Carotene	8·4	5·4	13·8	10·7	
Xanthophyll	25·6	45·0	25·4	47·3	

Hen No. 30: Date ...	24. iv.	4. v.	9. v.	27. v.	29. vi.	30. vi.	8. vii.–4. ix. Average of 3 yolks
Vitamin A	157·0	72·8	65·2	58·3	149·2	152·1	158·2
Carotene	9·8	4·2	2·8	12·3	12·4	7·6	10·8
Xanthophyll	41·6	24·0	32·0	32·8	58·8	53·0	43·7

III. Effect of dehydrated alfalfa

Hen No. 9: Date ...	30. v.	26. vi.	30. vi.	6. vii.	9. vii.–6. ix. Average of 4 yolks
Vitamin A	14·7	151·2	153·6	211·2	207·0
Carotene	3·4	22·0	15·8	11·8	27·8
Xanthophyll	42·0	237·6	192·6	146·0	304·0

Hen No. 3: Date ...	13. iv.	26. v.	5. vi.	22. vi.	26. vi.	2. vii.–7. vii. Average of 2 yolks
Vitamin A	149·7	76·8	35·8	119·5	174·0	159·6
Carotene	11·0	7·8	5·8	8·4	12·4	14·0
Xanthophyll	88·6	24·8	32·0	155·6	153·2	184·0

Hen No. 47: Date ...	18. v.	24. v.	26. v.	6. vi.	19. vi.	20. vi.	24. vi.	27. vi.–7. ix. Average of 4 yolks
Vitamin A	72·9	61·9	90·1	35·6	80·1	147·7	308·7	250·5
Carotene	2·8	3·6	7·6	6·5	15·0	9·4	18·0	38·9
Xanthophyll	28·6	44·2	37·4	31·4	66·5	133·8	324·8	310·5

* The dohyfral oil and cod liver oil were administered to the fowls separately.

In several cases the effects of the three supplementary foods showed very rapidly. A few examples will illustrate this point.

One of the dohyfral hens showed a rise of almost 50 units in 6 days; one of the alfalfa hens a rise of about 85 units in 8 days. The effects of cod liver oil showed up less quickly. Those that reacted most swiftly were the alfalfa hens. In this connexion we must not forget that the amount of carotene in lucerne is about $1\frac{1}{2}$ times that absorbed in the form of dohyfral and cod liver oil.

It should also be noted that 600 I.U. of vitamin A or an equivalent amount of carotene may be regarded as a fairly normal dose for a laying hen. Hence the amounts of dohyfral oil etc. here indicated should not be regarded as too high for these largely exhausted hens. This is also evident from the fact that the contents of these yolks did not equal those of the eggs derived from the best-fed groups even after the supplement was given for more than a month.

The vitamin A content of the eggs of the hens of group G without supplementary food was already in the middle of June considerably lower than that of eggs of the hens of the other groups that were regularly fed fairly well or very well.

Hens of group G that were given no supplementary food stopped laying when the yolks contained about 2 (or between 1 and 3) I.U. of vitamin A per g. The death rate among these fowls was high.

As the hens of group G consumed per day about 100 μg. carotene and about 25 I.U. vitamin A (contained in about 3·3 g. fish meal) and laid about 1 egg in 3 days, it is easy to calculate that only a small percentage of the sources of vitamin A were transferred to the eggs. The percentage in group G is smaller than in some of the other groups, for instance group B, where the consumption of carotene was about 660 μg. and of vitamin A about 50 I.U. Here the number of eggs is about 2 in 3 days, the yolks containing 320 I.U. of vitamin A and 31 μg. carotene, so that about 1/3 of the vitamin sources are transferred to the egg. This difference between groups G and B is not surprising if we assume that the organism itself needs a considerable amount of vitamin A per day. Supposing this is from 100 to 150 I.U., then group G has little or nothing left for secretion into the yolk, while in the case of the hens in group B there is enough left for the needs of the body even after a rich contribution has been made to the yolk.

One hen of group G (No. 45) went on laying longer than the others—until 31 July, in fact, on which date she was killed. From 6 April she had been fed on the group G diet and during that period had laid 68 eggs. The vitamin A content of these eggs gradually decreased to 70 I.U. or 3·6 per g. of yolk. Her liver proved to contain little vitamin A (5·93 I.U. per g. = 260 I.U. altogether).

The egg she was about to lay (its shell was already formed) still contained 54 I.U. of vitamin A, while the four largest yolks in the ovary contained a total of 73·5 I.U. of this vitamin, or 2·2 per g. Doubtless this hen, if it had been allowed to live, would only have laid a few more eggs at best. The body contained a great quantity—estimated at about 100–150 g.—of body fat of an intense yellow colour. Little vitamin A was found in this, only 3·8 I.U. per g. or a total of from 400 to 600 I.U.

To show that the results obtained from hen No. 45 are by no means unusual, we may report the following. In the 14 eggs examined before 13 July we found the average content to be 132 I.U. of vitamin A and only 9·4 μg. of carotene. The 68 eggs laid during the experimental period must have contained a total of about 8500 I.U. of vitamin A and 600 μg. of carotene. In the liver of the hens (at the beginning of the second laying) we repeatedly found great quantities of both substances, sometimes even more than 20,000 I.U. of vitamin A and more

than 1000 μg. of carotene (the average quantity of vitamin A of 7 livers from group A was 14,443 I.U.). The animal consumed about 25 I.U. of vitamin A and 100 μg. of carotene each day, that is in 4 months about 3000 I.U. and 12,000 μg. of these substances respectively, in other words more than appeared in the eggs. Hence it may be that this hen's liver contained enough of both substances at the beginning of the laying to supply the yolks and in part its own needs.

The carotene content of the yolks of the eggs of group G

Since the hens of group G, as long as supplementary rations (particularly alfalfa) were withheld, absorbed little carotene, the content of the latter was naturally low. It was less than 15 μg. per yolk. It is natural that a supplement in the form of shark's oil or cod liver oil produced very little increase. Of the carotene contained in the alfalfa a part was converted into vitamin A, another part found its way into the eggs. In the case of hens 9 and 47 this increased respectively from about 3 and 5 μg. per yolk to 27·8 and 38·9 μg. carotene. The carotene contents in yolks from the groups which were given during the whole experiment dehydrated alfalfa and yellow maize were generally of the same order.

The xanthophyll content of the yolks of group G was also very low on account of lack of yellow maize and lucerne. In May and June it was about 20 and 40 μg. Giving the hens dohyfral or cod liver oil produced no change worth mentioning, although the figures showed a certain increase in the case of a few of the birds. On the other hand alfalfa brought the content up to 300 μg. per yolk in the case of 2 of the 3 hens and to 200 μg. in the case of the third—values which correspond pretty well to those of the yolks from eggs belonging to various groups which were given yellow maize or lucerne throughout the whole experimental period.

From the results obtained in group G we may certainly conclude that yolks containing less than 60 I.U. of vitamin A per yolk (or about 3 I.U. per g.) are rare, because a diet as poor in carotene as that of group G will seldom be used, and furthermore the hens stop laying when the vitamin A content of the yolks is so much reduced. Hence these figures may be taken as the lowest limit for hens' eggs for sale on the market. For carotene this lowest figure is taken to be about 5 μg. or about 0·25 μg. per g. of yolk.

II. *Vitamin A, carotene and xanthophyll contents of yolks in the eggs of hens normally fed*

Turning from eggs laid by hens fed on unusual diets, we will now examine the results obtained in groups subsisting on rations used on different poultry farms.

We may class our groups under three heads:

(*a*) Groups A, B, V and VII, the diet containing yellow maize and alfalfa.

(*b*) Groups C, D, E and VI, the diet containing yellow maize but no alfalfa.

(*c*) Groups F and H, the diet containing alfalfa but no yellow maize.

The carotene and xanthophyll contents of the different rations were approximately as follows:

The mash fed to A and B contained 10·2, D and E 3·1, 7·6 and H 12·5 μg. carotene per g.

In the grain mixture containing yellow maize 1·8 μg. carotene per g. were found, in that containing white maize there was 0·25 μg. per g. Hence in 110 g. of the rations of A and B there were 561 + 99 or 660 μg. carotene. In 110 g. of group H diet there were 687·5 + 13·75 = 701 μg.

The 3 g. of fish meal which the hens consumed daily contained about 25 I.U. of vitamin A.

In the first months we analysed single yolks, later on we mixed yolks of eggs laid by hens belonging to the same group.

The differences of vitamin A content in the yolks from the different groups come out most clearly from a comparison of results obtained with mixed yolks. For the mixture we always used 10 yolks of hens belonging to the same group. In this way the individual differences were greatly reduced. The yolks in question were from eggs laid between 26 July and 6 September, i.e. all at a time when the hens had already had the same rations for more than 5 or 6 months respectively.

To enable the reader to compute from the contents of whole yolks, those of 1 g. of yolk, we may mention that the average weight of the mixed yolks of one set of groups was 18·76, that of the other was 17·8 g.

(*a*) *Vitamin A contents of the mixed yolks of eggs laid by groups fed on yellow maize and lucerne* (*the mash fed to group A also contained dohyfral*).

Groups ...	A	B	V	VII
	332·2	338·8	259·7	276·8
	332·2	305·1	271·1	309·3
	304·8	307·1	267·7	293·0
	327·3	312·9	270·4	290·6
	310·8	333·3	250·3	284·0
Average	321·4	319·4	263·8	290·7

About 320 I.U. per yolk were found in groups A and B. For group VII this figure was less than 300, while for group V it was considerably lower still, namely about 264. A comparison of the vitamin A contents of yolks (both singly and mixed) belonging to groups A and B respectively showed that there was little difference in spite of the fact that group A's rations contained dohyfral (about 100 I.U. vitamin A in a day's rations), which was absent from the diet of group B.

In contrast to this is the effect of dohyfral in the case of the exhausted hens of group G, the yolks of which showed a considerable increase of vitamin A when about 740 I.U. per day were added to the rations in the form of dohyfral oil.

The livers of the hens of groups A and B showed a difference of vitamin A content amounting to 5349 I.U. per liver. This difference is small when the amount of dohyfral oil consumed in the course of 9 months (about 27,000 I.U.) is considered, but it is presumably connected with the consumption of this latter substance by group A.

Concerning the difference (55·6 I.U.) between the yolks of groups B and V it may be mentioned that the only variable factor in these groups was the housing.

(*b*) *Vitamin A contents of yolks of eggs laid by groups fed on yellow maize and no alfalfa* (*the food of D and VI contained dohyfral as well*).

Groups ...	D	VI	E
	132·3	139·2	138·7
	152·9	146·3	149·7
	156·2	135·1	149·7
	152·0	147·4	146·3
	165·9	140·7	147·4
Average	151·9	141·7	146·4

As the figures of these tables show, a diet including yellow maize and alfalfa produced yolks containing twice as much vitamin A as rations without alfalfa.

Only once was a mixed yolk of group C analysed. It proved to contain 142·5 I.U. per yolk. Groups C, D and VI were fed alike. Group E differed from these three in that the hens received no dohyfral oil. The results showed that this difference did not affect the vitamin A content of the yolks.

(c) *The vitamin A contents of the yolks of the eggs from the groups fed on alfalfa and no yellow maize (the food of these groups contained no dohyfral).*

Groups ...	F	H
	157·1	254·8
	144·8	281·6
	159·0	267·0
	168·3	282·9
	170·6	259·9
Average	160·0	269·2

The difference between groups F and H in regard to diet lay in the fact that alfalfa constituted 10 % of the mash of group F and 15 % of that of group H, or about 5 and 7·5 % of the whole rations of these two groups respectively.

While the average content of group F corresponded with that of the groups that were given yellow maize but no lucerne, the content of the yolks obtained from group H was considerably higher than the latter, though distinctly lower than the content of those whose rations contained both yellow maize and lucerne. Hence 5 % lucerne in the mash or 2·5 % in the total daily diet given to group H over and above the quantity fed to group F had not quite the same effect as the 25 % maize in the case of group B, in spite of the fact that it contains more carotene. From a comparison of groups VII and H we may conclude that it is inadvisable to give hens maize in addition to 15 % of lucerne in the mash on account of the small difference effected in the vitamin A content of the yolks (290·7–269·2 = 21·5 I.U.).

In regard to the question whether 15 % alfalfa in the mash is not rather too much for other reasons, i.e. on account of the high percentage of crude fibre, we will only remark that in our opinion this is not unlikely.

The vitamin A contents of single yolks examined from the beginning of the experiment till about the end of July or the beginning of August were as follows.

When both alfalfa and yellow maize were given the content of vitamin A was somewhat more than 200 I.U. at the beginning of the experiment (groups A and B), which figure rose to a good 300 by the beginning of August (some yolks did not reach more than about 250–270[1]); groups V and VII did not rise to more than 230 and 250 I.U. respectively.

On a diet including yellow maize but no lucerne the yolks produced during May and June (groups C, D and VI) contained 150 I.U. of vitamin A. This amount corresponds with that of the mixed yolks of these groups.

No yolks from group E (yellow maize, no alfalfa or dohyfral) were examined in June and July. At the beginning of May they showed respectively 98·4, 123·1 and 122·8 I.U.

In group F given alfalfa without yellow maize or dohyfral the content of the single yolks (yolks of 4 hens were examined) rose to 224 I.U. before August (namely to 212·4, 247·4, 225·7 and 209·9). The average content of the August mixed yolks was 160 I.U.

In the case of 3 hens in group H the content of the yolks rose towards August respectively to 258·3, 243·8 and 261·2 I.U., which means that they reached about the same height as the mixed yolks in August.

[1] As the hens had not had any alfalfa before, a gradual increase was to be expected.

From the analyses of mixed yolks and single yolks we see that the vitamin A content of these yolks varies very much according to the food given to the hen. A diet that was very good from this point of view produced yolks with a content 5 or 6 times as high as that of the yolks with the lowest content; moderate rations produced yolks containing 3 times as much. The minimum contents were about 50 to 60 I.U. per g. of yolk, the maximum about 330. It appeared that they vary greatly with the amount of carotene in the food. A comparison of groups E and G proves that the carotene of yellow maize[1] (without lucerne and without dohyfral) increases the vitamin A content of the eggs. It also improves the egg production and health of the hens. A comparison of F with G shows the same effect of alfalfa (without dohyfral and without yellow maize). This effect is a little greater than that produced by yellow maize for, as we saw, the average of group F was 160, of group E 146·4 I.U. The carotene content of the food of group F was higher than that of group E.

The carotene and xanthophyll contents of mixed yolks in μg. per yolk

(*a*) *In the yolks of groups given a diet containing both yellow maize and dehydrated alfalfa the following amounts of carotene and xanthophyll* (*μg.*) *were found* (*the rations of group A also included dohyfral*).

	A		B		V		VII	
	Car.	Xan.	Car.	Xan.	Car.	Xan.	Car.	Xan.
	35·6	438·6	29·8	412·2	28·4	417·0	35·8	335·4
	44·4	524·6	32·2	394·4	31·1	341·0	34·4	360·0
	33·2	474·2	38·2	390·0	27·5	340·4	33·9	451·2
	38·2	535·6	32·0	388·0	23·2	262·0	36·0	471·1
	40·6	558·1	24·8	402·3	29·0	295·0	37·2	462·8
Average	38·4	506·2	31·4	397·4	27·8	351·0	35·4	461·1

Comparing the vitamin A content with the carotene content it may be seen that the number of I.U. of vitamin A was nearly 10 times the number of μg. of carotene. If we assume that 1 I.U. of vitamin A is equivalent to 0·6 μg. carotene, the ratio of the biological values is about 5 : 1.

The xanthophyll content was more than 10 times the carotene content. Hence the colour of the yolks produced on yellow maize and lucerne—and undoubtedly in general on green food—is chiefly due to xanthophyll.

To conclude from this that there is no reason to prefer eggs with highly coloured yolks to others is however not correct, for the intense colour is a fairly sure proof that sufficient green food was consumed by the hen and this green food is, as we saw, a good source of vitamin A in yolks.

An egg gets its value as a source of vitamin A almost exclusively from the vitamin itself.[2] We noted that this is also true of eggs of hens fed on a diet poor in vitamin A and carotene, as in the case of group G.

[1] The only difference between the foods of these two groups was that group G was given white and group E yellow maize.

[2] In saying this we take for granted that xanthophyll is of no value for the human or animal organism as a source of vitamin A.

(*b*) *In the yolks of eggs produced on a diet including yellow maize but no alfalfa the following amounts of carotene and xanthophyll* (*μg.*) *were found* (*the diet of groups D and VI contained also dohyfral*).

	D		VI		E	
	Car.	Xan.	Car.	Xan.	Car.	Xan.
	14·4	140·0	22·2	315·0	21·0	287·7
	17·4	177·6	24·0	298·0	18·2	280·0
	22·6	190·6	17·8	229·0	25·0	258·5
	22·8	149·1	16·8	277·2	25·2	291·0
	24·2	202·0	21·4	334·6	26·0	320·0
Average	20·3	171·8	20·4	290·7	23·1	287·4

As was to be expected the carotene content is lower here than in groups given both maize and lucerne. Here too, as in groups given both alfalfa and maize, the carotene content is small as compared with that of vitamin A; the ratio between the two is not quite the same as that found in the other groups; for in D, VI and E about 20 μg. of carotene were found, while each yolk was found to contain about 147 I.U. of vitamin A. Hence the ratio was about 1 : 7. In the case of groups A, B, V and VII it was nearly 1 : 10.

These yolks, too, are rich in xanthophyll. In groups VI and E the xanthophyll contents were respectively 14 and 12·5 times as large as the carotene content. Why group D eggs contain so much less xanthophyll per yolk cannot be explained; the rations of D and VI were identical, moreover these yolks derive their colour chiefly from xanthophyll. That maize can produce yolks with a high xanthophyll content is obvious from the figures of group E (without alfalfa or dohyfral).

(*c*) *Carotene and xanthophyll contents* (*μg.*) *of yolks from groups with rations containing alfalfa but no yellow maize* (*there was no dohyfral in these diets*).

	F		H	
	Car.	Xan.	Car.	Xan.
	18·4	240·0	22·2	327·0
	14·0	226·0	22·4	277·6
	18·2	268·0	25·6	242·0
	20·0	256·8	21·0	265·0
	15·0	280·8	19·6	316·4
Average	17·1	256·1	22·1	258·6

The carotene content in H is higher than in F, a fact which is not to be wondered at considering that the mash fed to H contained 15 % of lucerne, while that fed to F contained only 10 %. Maximal contents were not reached in these groups.

It appears that the carotene in yellow maize is a better source of vitamin A and carotene in yolks than that contained in lucerne. The xanthophyll contents in groups F and H are somewhat less than in groups E and VI.

Our results agree with those obtained by the biological method (experiments with young rats). Bethke *et al.* [1927] reported experiments in which the vitamin A of yolks was determined with young rats. It was found that: (1) vegetable food (e.g. blue grass) gave the same growth results as cod liver oil, and (2) the growth of the rats was satisfactory if they were given at least 0·1 ml. of yolk of egg, provided that the latter was laid by a hen fed cod liver oil, alfalfa or blue grass.

If by "satisfactory" was meant the growth accepted by Sherman-Munsell as the basis for the rate of growth, then we may conclude that the whole yolk

contained 180 Sh.M. units. But presumably Bethke and co-workers required a rate of growth greater than "satisfactory". If 1·4 I.U. were taken as equivalent to 1 Sh.M. unit (as is often assumed) then Bethke's yolks must have contained at least 250 I.U.

A third conclusion reached by Bethke was that when the animals were given a satisfactory diet the biological action of the yolks was five times as great as when the diet contained insufficient vitamin A and carotene.

Bearse & Miller [1937] concluded from their experiments with young rats that yolks contain 7·5–40 Sh.M. units per g., or per whole yolk 135–720. A higher content was obtained by means of a sufficient amount of dehydrated alfalfa.

Chicks hatched from eggs laid by hens which had consumed little carotene died sooner on a diet poor in vitamin A than chicks from better eggs. We found that the amount of vitamin A present in the liver of newly hatched chicks ran parallel with the content of the yolks. This result agrees with that found by Baumann *et al.* [1939], i.e. that the vitamin A content of the bodies of newly hatched chicks ran parallel with the number of units found spectrophotometrically in the yolks. The number of days these little chicks could be kept alive on a diet poor in vitamin A was also dependent on the amount of this substance found in the yolks by the non-biological method.

Hence we may conclude that the results obtained by the biological method correspond very well with our own, which fact is significant in establishing the value of our results, in view of the uncertainty which still exists as to whether physico-chemical estimation gives the same results as the biological method. Both methods may be used for the materials we are dealing with. We estimated the amounts of vitamin A and carotene in a large number of livers of our hens and young chicks: (*a*) from hens belonging to our 11 groups, (*b*) from chicks hatched from eggs laid by hens of groups V, VI, and VII and (*c*) from young cockerels fed on various diets. The results of these estimations corresponded with what we expected on the ground of our researches into the relation between yolks and diets.

It may be expected that hen's eggs on the market will contain about 300 I.U. of vitamin A. Poultry farmers are well aware that eggs for marketing are often not very good for hatching. The rations which poultry breeders usually give their hens are usually better than those used by farmers whose aim is to produce eggs for consumption.

It should be noted that the low content found in the case of group G is seldom met with, because the egg production of hens given this diet decreases too much and the death-rate is too high.

We may conclude that in eggs, as in milk and butter, the vitamin A activity depends very largely on the amount of green food consumed, whether fresh or conserved in such a way as to allow a considerable part of the carotene to be retained. The vitamin A activity of butter is of the same order of magnitude as that of yolks.

Where poultry have access to pasture they eat enough grass to bring up the vitamin A content of the yolks to a maximum. This undoubtedly is often not the case with poultry kept to produce eggs for consumption.

As we saw, feeding 25 % yellow maize as the only source of carotene produced a vitamin A activity in the eggs of our experiments only half as great as feeding both maize and green fodder (dehydrated alfalfa). In actual practice it is unlikely that poultry will often be given sufficient maize to produce eggs with 300 I.U. of vitamin A.

SUMMARY

The object of this research was to determine the vitamin A, carotene and xanthophyll contents of the yolks of hens' eggs of groups fed on different diets. After preliminary treatments both the yellow colour derived from carotene and xanthophyll and the blue colour (derived from vitamin A according to Carr and Price) were determined by means of the Stufenphotometer.

We used 300 hens for our experiments which lasted 9 months.

Although storage of vitamin A in the liver before laying begins, and in general individual capacities of the hens, may give rise to marked differences, it appeared that after the birds had been in production for some time the amount of the three above-named substances found in the yolks depends chiefly on feeding, i.e. on the vitamin A potency of the hens' rations. Hence it is this factor which determines whether a yolk contains 300 or 150 or only 50–60 I.U. of vitamin A. In the latter case the content could be distinctly increased within a few days by supplementing the diet with dohyfral (containing shark oil), cod liver oil or alfalfa.

Yellow maize and green fodder, either fresh or dried, are the most important sources of the vitamin A and carotene of the yolk. With the help of dehydrated alfalfa, cod liver oil or dohyfral oil, hens which had previously been reduced to a state of vitamin A exhaustion were brought into a condition where they laid eggs the yolks of which contained twice to four times as much vitamin A as before.

The highest values were found in eggs from hens fed on a diet containing both yellow maize and dehydrated alfalfa. Yellow maize alone—constituting 25% of the rations—produced yolks with a vitamin A content of 150 I.U. per yolk; with 7·5% alfalfa containing 140 μg. carotene per g. in the ration but without yellow maize it was about 270 μg. per yolk.

Yolks with the highest vitamin A content—produced on rations containing yellow maize plus lucerne—had a carotene content of almost 40 μg.; the poorest eggs fell to less than 10 μg. per yolk.

Hence a yolk derives its vitamin A potency mainly from the vitamin itself. The main source of the latter is, however, the carotene contained in the food.

There is much more xanthophyll than carotene in yolks. The yellow colour is therefore chiefly due to the former. Nevertheless strongly coloured eggs are generally also rich in vitamin A and carotene, because the xanthophyll in them is certainly in part derived from those components of the diet which also contain a great deal of carotene.

Green fodder plays a very important part in connexion with the vitamin A potency of eggs, as it does in the case of milk and butter.

Our results are in good agreement with those obtained by the biological method.

On several poultry farms the vitamin A potency of the ration is not sufficient for the production of eggs containing more than 150–200 I.U. of vitamin A.

Grateful acknowledgement is made to Dr J. Grashuis, Director of this Institute, for his interest in these researches and for having put at our disposal all that we needed to carry on our investigations; to Mr A. A. Haas for his analytical help in the laboratory and to Mr A. Versteege, foreman of the poultry department, for his care of the birds.

REFERENCES

Baumann, Semb, Holmes & Halpin (1939). *Poult. Sci.* **18**, 48.
Bearse & Miller (1937). *Poult. Sci.* **13**, 39.
Bethke, Kennard & Sassaman (1927). *J. biol. Chem.* **72**, 695.
Wolff (1938–9). *Z. Vitaminforsch.* **7**, 227.

91. SACCHARASE ACTIVITY IN THE BARLEY PLANT

By HELEN KEMP ARCHBOLD

From the Research Institute of Plant Physiology, Imperial College of Science and Technology, London, S.W. 7

(*Received 18 March 1940*)

It has long been recognized that most plant tissues have the capacity of inverting sucrose, but intensive study of saccharase action has hitherto been almost exclusively concerned with the saccharase of yeast [O'Sullivan & Tompson, 1890; Hudson, 1908, 1, 2; Michaelis & Menten, 1913; Nelson & Hitchcock, 1921; von Euler & Josephson, 1923; Willstätter & Kuhn, 1923; Weidenhagen, 1930, and many other papers by these authors and their collaborators]. The information regarding the saccharase of higher plants is frequently confined to records of its presence and of the optimum *p*H for its action [Kastle & Clark, 1903; Bailey, 1912; Colin, 1915; Maestrini, 1921; McGuire & Falk, 1920; Traegel, 1923; Kondo *et al.* 1929; Astruc & Mousseron, 1927; Blagoveschenski & Sossiedov, 1925; Matsusima, 1937]. Some attention has been paid to possible differences in the course of sucrose hydrolysis by saccharases from different plants [McGuire & Falk, 1923] and the effect of nutrient ions, particularly potassium, has been studied by Doby & Hibbard [1926; 1927], Harrt [1929] and Cattle [1933], while Vinson [1908] and Emiliani [1938] record variations of activity with age in the date and vine respectively. Finally a group of Russian workers [Arassimovitsch, 1939; Kursanov, 1936, 1, 2; Kursanov & Kriukova, 1937, 1, 2; Oparin, 1937, 1, 2; Rubin, 1936; Sisakyan, 1937] have quite recently investigated problems of saccharase action by infiltration methods.

The present paper records the changes in saccharase activity of the organs of the barley plant during the later half of the vegetative stage of growth and throughout the phases of stem elongation and ear development. The purpose of the investigation, which forms part of a study of the physiology of the barley plant in progress in this laboratory, was to ascertain whether the potential saccharase activity, as measured by the action of killed tissue on sucrose, could in any circumstances be a limiting factor in the interconversion of the sugars. It may be stated at once that no such evidence was found. Potential activity was always far in excess of all possible requirements, and the experiments merely emphasize the complexity of the problems involved in the mechanism of saccharase action. Nevertheless the results form a fairly complete record of the changes occurring in the barley plant with age, and for this reason it has been considered worth while to publish them.

The preliminary work necessary to ascertain appropriate conditions for making comparisons of activities at different times is first discussed.

Preliminary experiments on the saccharase of barley; methods of determining saccharase activity

Activity of the barley samples was determined by their action on a known amount of pure sucrose under standard conditions, the amount of sucrose inverted being estimated polarimetrically. Relative activity may be measured

by comparing either the initial velocities of inversion, or the time taken to invert a given, relatively large, percentage of the sucrose. The latter procedure has been adopted here as it requires fewer readings than the accurate determination of initial velocity. It was particularly necessary to minimize the number of observations to be made on each sample as it was found essential to use as inverting agent the ground up material rather than an extract. In these circumstances the usual method of removing aliquots from the digestion flask at appropriate time intervals was inapplicable, since the suspended solid matter could not be removed in proportion to the liquid. A separate sub-sample had therefore to be weighed out for every reading.

For the measurement of times taken to effect a given change Oppenheimer & Pincussen [1929] recommend observation of the times required for an inverting sucrose solution to reach zero rotation (75·93 % hydrolysis). Detailed figures have been published for the course of hydrolysis by yeast saccharase of a 4·75 % sucrose solution, maintained at 30° and *p*H 4·6, from which such times can be calculated if the angle of rotation be observed at some point between 30 and 70 % hydrolysis. On the arbitrary time scale given by these authors zero rotation is reached at time 13·55, and 50 % hydrolysis (1·88°) at time 7·10, while corresponding time factors are recorded for every 0·03 degrees. Thus if it is found that 50 % hydrolysis is reached in, say, 2 hr., the time taken to reach zero rotation is given by the equation $T=\frac{13\cdot55}{7\cdot10}\times 2=3\cdot8$ hr., and the saccharase activity will be inversely proportional to the values of T so found. Two readings are taken (preferably between 40 and 70 % hydrolysis) for each sample, and the values of T should agree, unless the course of hydrolysis is different from that reported by Oppenheimer & Pincussen for their yeast preparations. For yeast saccharase (British Drug Houses) Oppenheimer & Pincussen's values were confirmed.

It is further stated by the above authors that saccharase will withstand the temperature of liquid air, and even short exposures to −197°, without undergoing change. Freezing with solid CO_2 immediately upon gathering therefore offered a simple method of preserving the material. For the preliminary work extracts were preferred to whole tissue because of the greater convenience of manipulation, although less than half the total saccharase was removed by the method of extraction used. To prepare extracts the frozen tissue was coarsely ground, and placed at 1° overnight, then warmed to room temperature and the sap pressed out with a hand press. Aliquots (25–30 ml.) of the green colloidal suspension obtained were measured out into 100 ml. flasks and 10 ml. of acetate buffer added. The flasks were placed in a large water bath at 30°, and after 5 min. 50 ml. of a 9·5 % sucrose solution, previously warmed to 30°, were run in and the volume made up to 100 ml. and the flask shaken as quickly as possible. The time was noted when the pipette was half empty. After a suitable time interval 25 ml. of the digest were removed and run into 5 ml. of 2·5 % Na_2CO_3, and the time again noted when the pipette was half empty. This amount of alkali neutralizes the buffer and leaves sufficient excess to stop the enzyme action instantaneously [Hudson, 1908, 1]. After standing 10 min. to complete mutarotation the solution was polarized in a 2 dm. tube at 20°, using a sodium vapour lamp as a light source. This procedure only differs from that of Oppenheimer & Pincussen in that Na_2CO_3 was used to stop the reaction instead of NaOH, in order to avoid the risk of lowering the rotation of the remaining sucrose by excess alkali.

Clarification of barley extracts for polarization

After the addition of the buffer solution, the colloidal material gradually coagulated, leaving a clear brown supernatant liquid, whose colour was intensified by the alkali. As a result the colour was frequently too deep for satisfactory polarimeter readings. All extracts were therefore cleared with normal lead acetate; for this purpose the amount of Na_2CO_3 was added in a solution of such concentration that when a suitable amount of 20% lead acetate was used the volume of 25 ml. removed from the flask was still only increased to 30 ml. The addition of an amount of lead just insufficient to precipitate all the carbonate left, on filtration, a pale yellow liquid quite clear enough for polarization; addition of excess lead and its subsequent removal can therefore be avoided. To test whether sugar, and particularly fructose, was lost during clarification, sucrose solutions, hydrolysed by yeast saccharase, were examined at various stages of hydrolysis, and in addition observations were made on a barley extract sufficiently pale to allow of polarization without clarification. The results of these tests are given in Table 1, from which it is clear that the differences in the readings between cleared and uncleared solutions are negligible.

Table 1. *Rotation of partially hydrolysed sucrose solutions before and after clarification with normal lead acetate*

Initial concentration of sucrose in polarized solution 3·98%

(*a*) Hydrolysis by yeast saccharase			(*b*) Hydrolysis by extract of barley plants		
Time of reaction min.	Uncleared degrees	Cleared degrees	Time of reaction hr.	Uncleared degrees	Cleared degrees
0	5·24	5·25	20	3·18	3·19
15	1·46	1·52	20	3·22	3·19
30	0·50	0·51	20	3·25	3·25
45	−0·47	−0·49	20	3·29	3·22
1440	−1·64	−1·65	Mean	3·24	3·23

Uniformity of saccharase action

The problem of uniformity of saccharase action has been discussed by Nelson & Hitchcock [1921] who showed that for three samples of yeast saccharase the hydrolysis curves were superposable, while very slight differences were found for a fourth sample. On the other hand Nelson & Cohn [1924] found that for honey saccharase the course of hydrolysis for concentrations of sucrose below 4% was similar to that for yeast, but at higher concentrations there was an increase of velocity until 10 or 15% of the sucrose had been inverted. The optimum *p*H was, moreover, different from that of yeast. Ambard & Trautmann [1937] found that rates of sucrose hydrolysis by saccharases from man, rabbit, dog, snail, bee and yeast were all different, while Weidenhagen & Dey [1928] report differences in behaviour between taka-invertase and yeast invertase. McGuire & Falk [1923] observed an increase in the saccharase activity of banana extracts on standing of the order of 40–100%, which they show could not be due to spontaneous changes of *p*H. It cannot therefore be assumed that saccharases from all sources will behave in precisely the same way as that from yeast, and it became necessary to test the applicability of the Oppenheimer & Pincussen time factors to hydrolysis of sucrose by barley samples, more especially as the times taken to reach zero rotation when samples of reasonable size were used were far longer than the 2–3 hr. found for the yeast sample.

Using the procedure described above, including the lead clarification, two values of T, the time taken to reach zero rotation, were found for each of a number of barley extracts. In eight instances one pair of values is available from each extract, and in the other two a number of replicate observations were made. The results are set out in Table 2. The values of T ranged from 12 to 66 hr., and the mean difference between the pairs of observations was 1·2%. A statistical test (method of t for 20 pairs of observations gave $t=1$ [Fisher, 1938]) demonstrated that this mean difference was quite insignificant. In these samples therefore the course of hydrolysis of the sucrose was substantially the same as that reported for yeast by Oppenheimer & Pincussen, although it must be emphasized that small differences in the shape of the hydrolysis curve are not excluded. For example in sample 1 of Table 2, where the percentage hydrolysis at the first observation time (t_1) was only 21%, the values calculated for T are all higher than the corresponding values found at the second time (t_2) when 40% of the sucrose had been hydrolysed. This suggests that the rate of hydrolysis at the start is rather slower than the Oppenheimer & Pincussen curve demands.

Table 2. *Times taken to reach zero rotation in the hydrolysis of a standard sucrose solution by extracts of stems and leaves of barley at* pH *4·8*

Reading before hydrolysis 5·27°. Concentration of sucrose 3·98%.

t_1 and t_2 are the times (in hr.) from the start at which the samples were withdrawn from the digestion flask.

T is the time in hr. to zero rotation calculated by means of Oppenheimer & Pincussen's calibration table, see text.

Saccharase activity $\propto 1/T$

Sample	t_1	Angular rotation	Sucrose hydrolysed %	T	t_2	Angular rotation	Sucrose hydrolysed %	T
1	3	3·75	21·9	15·1	5·5	2·61	38·3	14·2
1	3	3·79	21·3	15·5	5·5	2·46	40·4	13·1
1	3	3·75	21·9	15·1	5·5	2·56	39·1	13·7
1	3	3·69	22·8	14·3	5·5	2·54	39·3	13·8
2	4·5	2·68	37·3	12·0	6·5	1·85	49·2	12·6
3	4·5	2·92	33·9	13·5	7·5	1·39	55·9	12·6
4	4·5	2·65	37·8	11·9	7·5	1·31	57·0	12·4
5	4·5	2·84	35·0	13·0	7·5	1·42	55·5	12·7
5	4·5	2·78	35·8	13·0	7·5	1·54	53·8	13·1
6	4·5	3·25	29·0	16·3	9·0	1·50	54·3	15·6
7	4·5	3·46	26·1	17·9	9·0	1·86	49·1	17·7
8	5·5	3·52	25·2	21·9	10·5	1·94	47·9	21·3
9	24	2·17	44·6	51·9	30	1·58	53·2	53·4
9	24	1·97	47·4	48·3	30	1·39	55·9	50·6
10	20	2·97	33·1	61·7	40	1·20	58·6	62·5
10	20	3·05	31·9	64·5	40	1·18	58·9	62·2
10	20	3·08	31·5	65·6	40	1·24	58·0	63·5
10	20	3·09	31·4	65·9	40	1·18	58·9	62·2
10	20	3·01	32·5	63·0	40	1·28	57·4	64·4
10	20	3·02	32·5	63·4	40	1·26	57·7	63·9
				Mean values of T 33·19				32·78

Mean difference 1·2%.

Value of t for 20 observations = 1 (P = 0·05).

Possible deviations from the course of hydrolysis found for yeast saccharase indicated in this way require confirmation by detailed study of the early stages of hydrolysis, but it seems clear that any such differences are not sufficiently

great to preclude the use of the calibration table for the purpose of comparing saccharase activities of barley samples provided that the reaction is allowed to continue until at least 30% of the sucrose has been hydrolysed.

The optimum pH for barley extracts

The determinations of saccharase activity discussed here have all been made at the optimum *p*H, and therefore represent the maximum potential inverting capacity. The actual capacity will be governed among other factors by the buffer system of the tissues. Plant saccharases have optimum *p*H values centring round 5. Thus Blagoveschenski & Sossiedov [1925] found a range of 4·5–6·2 for 14 angiosperms using ground air-dried leaves as the inverting agent, while Matsusima [1937] quotes 4·5–5 for tobacco leaves, and McGuire & Falk [1920] 4–5 for potato extracts. The activities of four separate extracts of barley leaves and stems for the range of *p*H 3·6–6·4 have now been found, two values of T being calculated for each extract at each *p*H. All four extracts showed optimum activity between *p*H 4·6 and 4·8 with little change up to *p*H 5. The results are shown graphically in Fig. 1. Fig. 1 shows for a single extract the mean values of

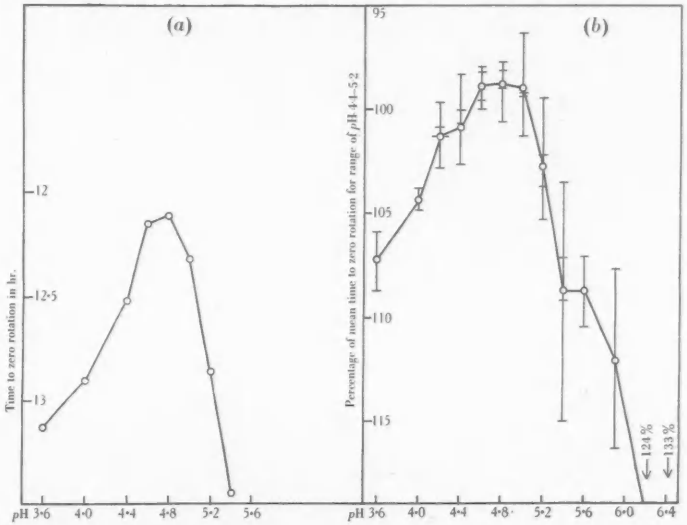


Fig. 1. Effect of *p*H on the saccharase activity of extracts of barley leaves and stems. (*a*) Saccharase activity of a single extract expressed as the time (in hr.) taken for a standard sucrose solution to reach zero rotation. (*b*) Mean saccharase activity of four extracts expressed as percentages of the mean time to zero rotation for the *p*H range 4·4–5·2.

T plotted against *p*H, and in Fig. 1*b* all the results are combined by calculating the activities (times to zero rotation) as percentages of the mean values for the range *p*H 4·4–5·2. The mean values for each of the four extracts are marked on the vertical lines, together with the means for all extracts. The shape of the curve is very similar to that found for yeast saccharase; it has the same flat top over the range 4·6–5·0, with a rather sharp fall on either side, but especially on the alkaline side of 5·0. The change in activity up to *p*H 6·4 is about 25%

of the maximum value. For the present experiments an acetate buffer at *p*H 4·8 has been used throughout. A few comparisons were made between the activities of unbuffered extracts and those buffered to 4·8; in all cases the unbuffered extracts showed a lower activity than those at *p*H 4·8, and corresponded approximately to those buffered to between 5·2 and 6. The expressed activity will thus be lower than the potential activity because of the *p*H, but from the curves of Fig. 1 it is apparent that over the rather wide *p*H range 3·6–6·4 this effect is only of the order of 25 %. The results of the comparison are shown in Table 3.

Table 3. *The saccharase activity of extracts of barley leaves and stems buffered to* p*H 4·8 and unbuffered*

Activity $\propto 1/T$

Sample	Time to zero rotation (T) in hr. At *p*H 4·8	Unbuffered
1	12·68	17·91
2	13·03	17·57
3	12·33	13·43

Changes in saccharase activity with standing

In view of Falk & McGuire's [1920] findings on banana extracts, barley extracts were allowed to stand for various times to study the effect on saccharase activity. Only very small changes were found and there was no evidence of a similar situation to that found in the banana. From the data collected it appears that in barley there is a slow fall with time of standing, which is considerably retarded by low temperature, but that extracts can be kept at least 24 hr. at room temperature without measurable loss of activity. A similar slow loss of activity on standing has been found for potato leaf extracts [Doby, 1915]. As a result of these findings, the samples for routine determinations were generally thawed the evening before and kept at 1° overnight. The results of the experiments on time of standing are given in Table 4.

Table 4. *Effect of standing on the saccharase activity of 25 ml. aliquots of extracts of barley stems and leaves*

Activity $\propto 1/T$

Sample 1		Sample 2	
Hr. at room temperature	Times to zero rotation (hr.) T	Hr. at room temperature	Times to zero rotation (hr.) T
0	15·6	1	14·7
24	15·9	2	14·4
120	17·8	3·5	14·0
4 days at 1°	15·8	24	14·1

The use of whole tissue as the inverting agent

It was noted by Vinson [1908] that extraction methods failed to remove all the saccharase of dates and that the amount removed increased with the age of the fruits. Falk & McGuire [1920] made a similar observation for bananas, and Mason [1920] has drawn attention to the effects of different methods of extraction on the saccharase of *Galanthus nivalis*. The two former authors suggest that two types of saccharase were present, one soluble (free) and the other insoluble (bound) and that the former increased at the expense of the latter with

age. Grassmann [1928] summarizes the position as regards yeast and makes it clear that only about 10% of the total saccharase goes into a water extract, unless preliminary treatment with proteolytic enzymes, amylases and tannase, is employed, or the yeast is so finely ground that no distinct cell structure remains. He expresses the view that the difference in the state of the saccharase is one of adsorption rather than of the type of enzyme. These observations seem to have been overlooked by other workers on plant saccharases who have used extracts of dried or crushed tissues, or powdered dry material without a satisfactory demonstration of the effectiveness of the extraction methods used [Blagoveschenski & Sossiedov, 1925; Doby & Hibbard, 1927; Harrt, 1929; Cattle, 1933]. It is manifestly impossible to attempt the complete removal of saccharase from a large number of samples, and therefore whole tissue has been used in the present work. This procedure appears to be justifiable for experiments of the present type since Willstätter *et al.* [1922] and Willstätter & Kuhn [1923] have shown that the activity of the enzyme is not affected by purification. Some figures showing the relative amounts of enzyme expressed in the sap from frozen barley plants and that remaining in the press residue, together with a similar experiment on air-dried, ground grass samples are given in Table 5. Only about 1/3 of the saccharase was extracted after fine grinding, and the results are therefore in agreement with those for yeast.

Table 5. *Saccharase activities of sap expressed from duplicate 40 g. samples of frozen barley plants, and of water extracts of 5 g. samples of air-dried grass*

	Expressed sap	Washings after pressing	Residual tissue
	Time (hr.) to zero rotation, T		
Barley 1	52·6	151	18
Barley 2	49·9	138	19
	Water extract		
Grass	—	85	26

In order to deal satisfactorily with a large number of samples some method of preservation was required, as all could not be analysed immediately after collection. The choice lay between drying the samples or preserving them in a frozen condition. A trial of these two methods yielded the results shown in Table 6. The highest activity was found in the frozen samples and there was some loss even when drying was carried out at room temperature, while short exposures to 80° destroyed a large part of the saccharase. Accordingly all samples were preserved in the frozen condition.

Table 6. *The effect of drying on the saccharase activity of grass samples*

(*a*) Not dried. Tissue frozen and ground.
(*b*) Dried in air at room temperature.
(*c*) Dried at 80° for 1 hr.

(*a*)		(*b*)		(*c*)	
Time of reaction hr.	Sucrose hydrolysed %	Time of reaction hr.	Sucrose hydrolysed %	Time of reaction hr.	Sucrose hydrolysed %
1·1	2·4	—	—	—	—
4	5·0	—	—	—	—
20	41·8	19	28·8	20	2·7
		43	49·4	47	5·0
76	95	67	73	94	46·9

No change was observed in the activity of material kept frozen with solid CO_2 for 3 months. Some data bearing on this point are shown in Table 7. The mean difference between the values for the longer as compared with the shorter times of storage is negligible, but in some individual instances the differences are rather large.

Table 7. *Saccharase activity of barley leaves after storage in a frozen condition (solid CO_2)*

Activity $\propto 1/T$

Sample	Days between freezing and estimation	Time to zero rotation (T) hr.	Days between freezing and estimation	Time to zero rotation (T) hr.
1	15	11·9	73	11·2
1	15	11·8	73	10·7
2	22	12·7	71	10·8
3	20	13·0	69	13·5
4	65	14·6	91	14·7
5	44	13·9	88	16·3
5	44	13·5	88	15·0
	Mean	13·1		13·3

The results of these preliminary experiments may be summarized as follows. Hydrolysis of sucrose by barley saccharase follows substantially the same course as that by yeast saccharase, although small differences in the hydrolysis curve are not excluded. The optimum *p*H for barley saccharase lies between 4·6 and 4·8 and activity falls sharply beyond the values 4·4 and 5·2. Sap expressed from frozen tissue shows a slow decline in activity at room temperature detectable after about 24 hr. standing. When activities at different stages of plant growth are to be compared the whole tissue must be used as the inverting agent, and not an extract therefrom, since a small and variable part only of the saccharase is removed by any simple extraction procedure. Samples may be successfully preserved in the frozen condition by means of solid CO_2; this method is recommended in preference to any type of drying since there is a loss of activity even after air drying.

Seasonal changes in the saccharase activity of the barley plant.
Collection of material

The barley used for this investigation was a pure line var. Plumage Archer. The plants were grown in soil in pots in the open at Rothamsted Experimental Station during the summer of 1938. The seeds were sown on 13 May and after germination a dressing of nitrate was given. At the third leaf stage the plants were thinned to three per pot. Collections, begun at 10.30 a.m., were made from the time of emergence of the sixth leaf (27. vi. 38) until the end of September (27. ix. 38), when the plants were ready for harvest. Duplicate samples, usually of three plants, were taken on each occasion. The aerial parts were removed from the roots and weighed, divided into leaves, leaf sheaths, ears and separate internodes of the stem and the fresh weight of each component recorded. Duplicate 5 g. samples of each component were then weighed out and placed in tins (4 in. $\times$ 1½ in.) and the contents frozen instantaneously by placing the tins on a block of solid CO_2. The roots were washed out of the soil and similarly treated. Parallel samples were weighed out for the determination of dry weight. The samples were transported from the field in a container cooled with CO_2 and finally stored in similarly cooled, insulated chests till required. The frozen material was then removed into a previously cooled mortar and ground to a

powder. The powder, still frozen, was transferred to wide-mouthed, stoppered bottles, and allowed to thaw. If the mortar is cold enough to prevent thawing during grinding, only traces of material adhere to its surface and the losses on transference are negligible. Young tissues can be fairly readily ground in this way, provided that the mortar is first thoroughly cooled in the CO_2 chamber. Difficulties were experienced with older tissues, especially stems, and it is recognized that there is room for much improvement in the disintegration technique, since inefficient grinding reduces the inversion rate at least at the start. Some of the rather large variability found between duplicate determinations may have arisen from this cause, and where inversion rate is very slow a further source of error may be an actual fall in activity during the observation.

When the contents of the bottles reached room temperature 16 ml. of water (on the assumption that each 5 g. sample contains 4 ml. of water) and 5 ml. of buffer solution (*p*H 4·8) were added. After warming to 30°, 25 ml. of 9·5 % sucrose at the same temperature were run in, making a total volume of 50 ml. After an appropriate time at 30°, 25 ml. of liquid were removed from each bottle and run into 3·5 ml. of 3·5 % Na_2CO_3. After 10 min. 1·5 ml. of 20 % normal lead acetate were added and the solution filtered and "polarized". Blanks were run for each sample on a parallel 5 g. sub-sample, adding a further 25 ml. of water instead of the sucrose solution.

Experimental results and discussion

The results obtained for the 5 g. samples of fresh material for the several components of the barley plant are shown in Table 8. The saccharase activity is expressed as the reciprocal of the time taken for a standard sucrose solution

Table 8. *Saccharase activities of component parts of the barley plant during growth*

Saccharase activity per 5 g. fresh weight expressed as reciprocal × 10 of time (hr.) taken for a fixed amount of sucrose to reach zero rotation under standard conditions (see text, pp. 756, 757).

Date 1938	Stage of plant growth		Leaves	Leaf sheaths	Ears	Stems	Roots
27. vi.	6th leaf expanded	Leaf development	1·21	1·07	—	—	—
4. vii.			0·72	1·13	—	—	0·38
11. vii.			0·47	1·02	—	—	0·47
18. vii.			0·75	0·51	—	0·41	0·32
20. vii.	10th leaf expanded	Stem elongation	0·68	0·54	0·23	0·26	0·36
22. vii.			0·91	0·50	0·46	—	0·34
25. vii.			0·83	0·37	—	0·25	0·29
27. vii.	Awns showing		0·80	0·39	1·29	0·30	0·40
29. vii.			0·74	0·29	—	0·24	0·36
2. viii.	Ear emergence		0·73	—	1·64	0·25	0·27
4. viii.			0·71	0·42	3·33	0·21	0·32
6. viii.	3 green leaves left		0·70	0·65	2·33	0·22	0·40
10. viii.			0·56	0·55	1·72	0·17	0·41
16. viii.			0·78	0·55	0·76	0·20	0·36
22. viii.			0·65	0·68	0·43	0·14	0·22
29. viii.			0·86	0·49	0·33	0·18	0·18
5. ix.	All leaves dead	Ear development	—	—	0·27	0·19	0·23
12. ix.		Maturation without growth	1·16	0·51	0·22	0·18	0·18
19. ix.			1·52	0·54	0·20	0·11	0·26
27. ix.			1·28	—	—	0·21	0·25

to reach zero rotation under the conditions described. All parts of the plant showed considerable saccharase activity throughout the period studied, and although there was a general decline towards harvest, death of the tissues was

not accompanied by disappearance of enzyme. This decline was least in the leaves, where on a concentration basis dead leaves actually showed higher values than green ones, although the absolute amounts per leaf were the same. The occurrence of saccharase in the roots may perhaps be emphasized, since neither Colin [1915] nor Leonard [1938] found any in the contrasting type of storage root of beet.

In terms of the fresh weight the level of activity is lowest in the stems, with rates only slightly higher in the roots, and in both cases there is a decline to a more or less constant value soon after ear emergence (16. vii. 38). In the ears,

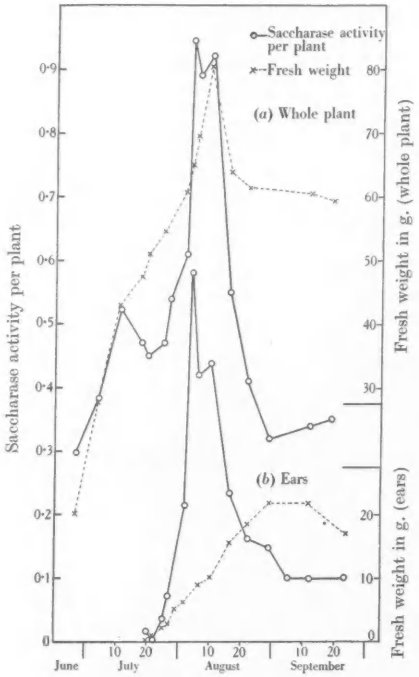


Fig. 2.

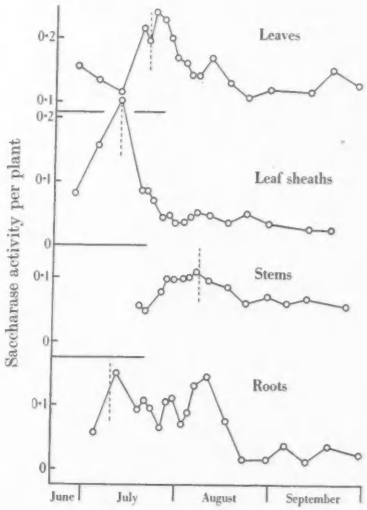


Fig. 3.

Fig. 2. Change in saccharase activity of the barley plant from the time of emergence of the sixth leaf until harvest. Saccharase activity expressed as the reciprocal × 10 of the time (in hr.) taken for a standard sucrose solution to reach zero rotation. (*a*) Total activity of whole plant. (*b*) Activity per plant of the ears. Dotted lines show corresponding fresh weights.

Fig. 3. Change in saccharase activity, per plant, of the leaves, leaf sheaths, stems and roots of the barley plant from the time of emergence of the sixth leaf until harvest. Saccharase activity expressed as reciprocal × 10 of the time (in hr.) taken for a standard sucrose solution to reach zero rotation. Dotted vertical lines show position of maximum fresh weight of each component.

activity rises sharply until emergence and then falls at first rapidly and later more slowly, reaching a final low level comparable with that of the stems and roots. The saccharase activity developed by the ear as it emerges is three times

as great as that shown at the same time by any other part of the plant, but, as will be shown later, it is likely that the high value is an expression of the ear's relative immaturity rather than of a greater capacity to form saccharase. In the leaves and sheaths the values are about twice as great as those of the stems and roots, and again activity tends at first to fall with time, but about the end of July for sheaths, and end of August for leaves, there is a secondary rise. This rise is coincident with rapid desiccation and death and only indicates the high rate of water loss relative to the slow loss of saccharase.

Figs. 2 and 3 show the saccharase activity of the whole plant and of its components calculated as the amounts per plant from the data of Table 8. In Fig. 2 the fresh weights are also included. As growth proceeds there is a rise in the absolute activity of the whole plant (Fig. 2*a*), but after the maximum weight is reached there is a rapid decline, largely due to changes in the ear (Fig. 2*b*) accompanied by only small losses of weight. In the case of the various parts (Fig. 2*b* and Fig. 3) a similar sequence of changes is found in the leaves, sheaths and stems, namely a rise to a maximum as each reaches its maximum weight (marked by dotted line in Fig. 3) followed by a fall to a more or less constant low value. In the roots the time of the maximum weight is doubtful and was probably prior to the start of the collections. Only the general decline is therefore indicated with certainty; moreover, too much weight must not be placed on the rather sudden drop at the end of August, since this is due to a similar fall in fresh weight, which was mainly brought about by increasing brittleness of the roots resulting in very imperfect removal from the soil at this stage. In the ear the maximum value occurred rather earlier than the maximum weight. This difference is doubtless due to the fact that the later increase in weight is largely the result of starch storage and not of cell division or expansion. Fig. 2 also emphasizes that the ear as it develops is contributing a rapidly increasing proportion of the total activity, for at this time all the other parts have passed their maximum weights, and saccharase activity is declining. The percentage contributions of the several parts as each reaches its maximum weight are shown in Table 9. At first nearly half the total amount is contributed by the leaves, but later the contribution of the ear rises to 60 %, while that of the stems is throughout of the order of 10 %.

Table 9. *Percentages of the total saccharase activity contributed by the various parts of the barley plant*

T is the time in hr. taken to reach zero rotation under standard conditions

At the stage of maximum fresh weight of	Total activity $1/T$	% of the total activity (round numbers)				
		Leaf	Leaf sheaths	Ear	Stem	Root
Root (18. vii. 38)	0·472	45	18	0	12	25
Leaf (20. vii. 38)	0·452	44	12	4	10	30
Stem (10. viii. 38)	0·893	16	6	47	12	19
Whole plant (10. viii. 38)	0·920	15	5	60	10	10
Ear (29. viii. 38)	0·414	29	8	35	17	11

Since the total saccharase content increases at approximately the same rate as that of the ear during ear development, while changes in other components are both relatively and absolutely small, there is no evidence of enzyme transference from one part of the plant to another; the conclusion to be drawn is rather that new enzyme is synthesized in each successive growing region.

In terms of the dry weight (Fig. 4), the early values are high in every instance, and there follows a fall at first rapid and then slow. The low level reached by the

stems and ears is less than that of the other components, probably as the result of the high proportion of structural tissues in the stems and of starch in the ear. Increase in saccharase activity relative to dry weight was only recorded for the ear, but in the other parts the very early stages of development are not included. It is likely that at correspondingly early stages of development the leaves, leaf sheaths etc. would show a corresponding rise, especially as the earliest recorded result for sheaths (7th leaf stage) in which the unexpanded leaves are included is of the same order as that of the ear as it reaches its maximum value. Values for the separate internodes of the stem are shown in Fig. 5.

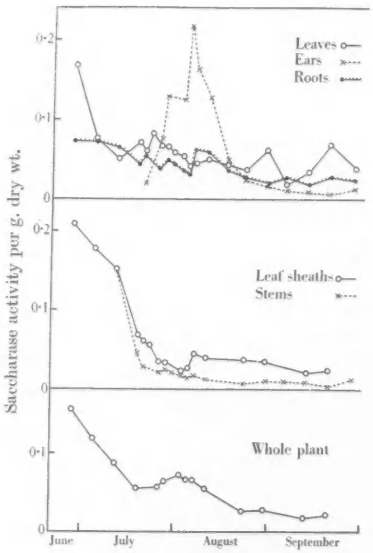


Fig. 4.

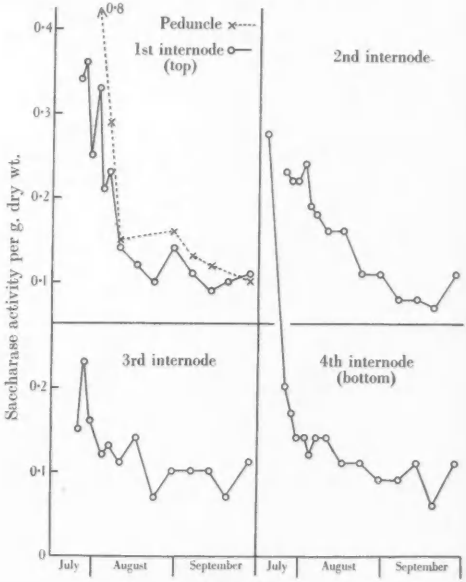


Fig. 5.

Fig. 4. Change in saccharase activity per g. dry weight of the barley plant and its components from the time of emergence of the sixth leaf until harvest. Saccharase activity expressed as the reciprocal × 10 of the time (in hr.) taken for a standard sucrose solution to reach zero rotation.

Fig. 5. Change in saccharase activity per g. dry weight of the stem internodes of the barley plant. Saccharase activity expressed as the reciprocal × 10 of the time (in hr.) taken for a standard sucrose solution to reach zero rotation.

The level of activity is about the same for them all and there is no indication of a gradient along the stem. The large top internode has a high initial value, due to its relative immaturity at the first analysis (18. vii.), and all show the decline in activity towards harvest. There is evidence that high values occur early in the development of the bottom (4th) internode and of the peduncle, and the same is no doubt true of the intermediate ones.

From the above account it would seem that saccharase formation is very closely associated with growth, in the sense of cell division and expansion, and in all probability especially the former. Consideration of the absolute amounts

present show that saccharase formation ceased immediately growth was complete, and this was true not only for the plant as a whole, but for each component as the succession of maximum weights was reached. The continuous fall relative to dry weight emphasizes the relatively high rate of production in the early stage of growth, and the course of saccharase accumulation appears to be very similar to that already well established for nitrogen, although nitrogen can of course be transferred from one part of the plant to another.

The results reported here are in general agreement with what is already known of saccharase changes. Thus Doby & Hibbard [1927] found a higher saccharase content in young than in old beet leaves, and Cattle [1933] found, in terms of leaf area, a falling activity from the first to the eighth leaf of bean plants comparable with the fall here noted with age in terms of the dry weight.

If potential saccharase activity be considered in relation to the supply of assimilate to the various parts of the plant it becomes apparent that the maximum possible requirements can always be met. For consideration of the saccharase activity in this relation it is necessary to make an estimate of the initial velocities of inversion. These have been calculated on the assumption that sucrose is hydrolysed according to the Oppenheimer & Pincussen curve, an assumption which has been shown to be sufficiently close to the facts to ensure that the calculated velocities are certainly of the right order. The maximum possible requirements have been calculated from the dry weight values on the assumption that all the assimilate arrives at (or departs from) any given organ as sucrose, and that it must be hydrolysed before it is further metabolized. Dry weight increases (or decreases) per hour per plant will then give a measure of the requirements.

Table 10. *The initial velocities of hydrolysis of sucrose by the component parts of the barley plant (after freezing and grinding), and the rates of change of dry weight (i.e the maximum possible amount of sucrose to be hydrolysed) during various periods of the growth cycle*

	Period 1938	Average rate of increase or decrease* of dry weight	Initial velocities of inversion at *p*H 4·8	
		mg. per plant per hour		
			Min.	Max.
Leaves:				
Dry weight increasing	27. vi.–29. vii.	22·7	192	244
Dry weight decreasing rapidly	29. vii.–10. viii.	25·5	208	209
Dry weight decreasing slowly	10. viii.–19. ix.	4·2	188	208
Stems:				
Dry weight increasing	18. vii.–10. viii.	10·1	61	118
Dry weight decreasing	10. viii.–19. ix.	2·5	73	86
Ears:				
Dry weight increasing rapidly	25. vii.–29. viii.	10·2	90	721
Dry weight increasing slowly	29. viii.–19. ix.	2·0	94	94
Roots:				
Dry weight increasing	22. vii.–2. viii.	5·0	110	148
Dry weight decreasing	2. viii.–29. viii.	2·0	54	54

In leaves and roots only a part of the phase of increasing dry weight is included, as observations were not begun until the emergence of the sixth leaf.

The calculations are brought together in Table 10, which gives the rates of change of dry weight for different periods of growth, and the range of initial velocities of inversion given by the samples of material collected during the same

period. Everywhere the velocities of inversion are many times as great as the rate of dry weight change, and therefore all possible sucrose could at all times be readily inverted by all parts of the plant. Accumulation of sucrose is thus never influenced by lack of potential inverting capacity, and it is quite clear that the presence of free sucrose presupposes some mechanism either chemical or physical preventing the exercise of the large inverting capacity. Kursanov [1936, 1] indicated in a general way the existence of some such system by his observation that the activity of living chicory (found by infiltration methods) was only 1/8th of that of the autolysed tissue, while Weidenhagen & Paa Chung Lu [1936] gave data showing the inhibiting effect of ascorbic acid, and Doby & Hibbard [1926] show that Cl ions may paralyse saccharase. From the present data it may be stated that it is extremely unlikely that an unfavourable *p*H plays an important part in the inhibiting system unless very large local differences of *p*H exist in the cell. It will be remembered that unbuffered barley extracts showed an activity similar to that found for a *p*H range of 5·2–6·0, while the fall in activity over the range 3·6–6·8 was of the order of 25 %. Reduction of the velocities given in Table 10 by even 30 % would still leave an inverting capacity largely in excess of that required for the incoming assimilate.

Summary

Saccharase activity has been determined in the component parts of the barley plant from the time of emergence of the sixth leaf until harvest.

Fresh material was frozen by means of solid CO_2, and the material stored in the frozen condition until required; activity remained unchanged under these conditions. Activity was measured as the reciprocal of the time taken to effect 75·93 % hydrolysis (i.e. the time to zero rotation) of a given amount of sucrose under standard conditions, the amount of sucrose inverted being found polarimetrically. It was necessary to clear the solutions with normal lead acetate before polarization.

Tissue, previously ground in the frozen condition, was used as the inverting agent as no simple extraction procedure removed more than a fraction of the saccharase. Drying even at air temperature caused some loss of activity.

The optimum *p*H for the saccharase of the barley plant was found to be 4·8, and all measurements were made at this *p*H.

In terms of fresh weight the level of activity was lowest in the stems, slightly higher in the roots, while leaves and leaf sheaths were about twice as active as the stems. In the ear activity rose rapidly with growth and declined again after full emergence. The maximum value in the ear was the highest recorded for any part of the plant.

The total activity of the plant rose until ear emergence, which was coincident with the maximum weight of the plant, and then declined. Each part showed a similar trend of increasing activity with its growth, followed by a fall. Loss of activity with time was least in the leaves, dead leaves being still quite active.

Expressed in terms of the dry weight the activity was high initially, and fell off at first rapidly and then more slowly. The differences in level between the various parts were small, stems and ears showing rather lower values than the other parts of the plant towards harvest, probably owing to the large proportion of structural tissue in the former and storage of starch in the latter.

Saccharase production thus appears to be associated with growth and to cease when growth ceases. No evidence was found of transport of enzyme in the plant. The production is relatively greatest in the very early stages of the

development of each organ, as is shown by the continuous fall in terms of dry weight.

The potential inverting capacity is always many times as great as the maximum requirement (measured by dry weight increase) in any part of the plant. The existence of free sucrose therefore requires the presence of some inhibiting mechanism. An unfavourable *p*H is unlikely to be a major factor in this mechanism since over the range *p*H 3·4–6·8 the reduction in activity as compared with that at the optimum value is only of the order of 25%.

REFERENCES

Ambard & Trautmann (1937). *C.R. Soc. Biol., Paris*, **125**, 133.
Arassimovitsch (1939). *Brit. chem. Abstr.* **33**, 871.
Astruc & Mousseron (1927). *C.R. Acad. Sci., Paris*, **184**, 126.
Bailey (1912). *J. Amer. chem. Soc.* **34**, 1706.
Blagoveschenski & Sossiedov (1925). *Biochem. J.* **19**, 350.
Cattle (1933). *New Phytol.* **32**, 364.
Colin (1915). *C.R. Acad. Sci., Paris*, **160**, 777.
Doby (1915). *Biochem. Z.* **71**, 495.
—— & Hibbard (1926). *Biochem. Z.* **178**, 139.
—— —— (1927). *J. biol. Chem.* **73**, 405.
Emiliani (1938). *Ann. Chim. appl. Roma*, **28**, 87.
v. Euler & Josephson (1923). *Ber. dtsch. chem. Ges.* **56**, 446.
Falk & McGuire (1920). *J. gen. Physiol.* **3**, 595.
Fisher (1938). Statistical Methods for Research Workers, 7th ed. p. 126. London.
Grassmann (1928). Neue Methode und Ergebnisse der Enzymforschung, p. 65. Munich.
Harrt (1929). *Bot. Gaz.* **88**, 229.
Hudson (1908, 1). *J. Amer. chem. Soc.* **30**, 1160.
—— (1908, 2). *J. Amer. chem. Soc.* **30**, 1504.
Kastle & Clark (1903). *Amer. chem. J.* **30**, 421.
Kondo, Nakajima & Suzuki (1929). *Brit. chem. Abstr.* **23**, 5162.
Kursanov (1936, 1). *Biokhimiya*, **1**, 269.
—— (1936, 2). *Biokhimiya*, **1**, 411.
—— & Kriukova (1937, 1). *Biokhimiya*, **2**, 674.
—— —— (1937, 2). *Biokhimiya*, **2**, 720.
Leonard (1938). *Amer. J. Bot.* **25**, 78.
Maestrini (1921). *Brit. chem. Abstr.* **15**, 379.
Mason (1920). *Sci. Proc. R. Dublin Soc.* **16**, 83.
Matsusima (1937). *Brit. chem. Abstr.* **31**, 5002.
McGuire & Falk (1920). *J. gen. Physiol.* **2**, 215.
—— —— (1923). *J. Amer. chem. Soc.* **45**, 1539.
Michaelis & Menten (1913). *Biochem. Z.* **49**, 333.
Nelson & Cohn (1924). *J. biol. Chem.* **61**, 193.
—— & Hitchcock (1921). *J. Amer. chem. Soc.* **43**, 2632.
Oparin (1937, 1). *Biokhimiya*, **2**, 135.
—— (1937, 2). *Enzymologia*, **4**, 135.
Oppenheimer & Pincussen (1929). Die Methodik der Fermente, p. 933. Leipzig.
O'Sullivan & Tompson (1890). *J. chem. Soc.* **57**, 834.
Rubin (1936). *Biokhimiya*, **1**, 467.
Sisakyan (1937). *Biokhimiya*, **2**, 687.
Traegel (1923). *Z. dtsch. Zuckerindustr.* **73**, 158.
Vinson (1908). *J. Amer. chem. Soc.* **30**, 1005.
Weidenhagen (1930). *Fermentforschung*, **11**, 154.
—— (1932). *Z. dtsch. Zuckerindustr.* **82**, 992.
—— & Dey (1928) *Z. dtsch. Zuckerindustr.* **78**, 242.
—— & Paa Chung Lu (1936). *Z. Wirtschaftsgruppe Zuckerindustr.* **86** (Tech. Tl), 240.
Willstätter, Graser & Kuhn (1922). *Hoppe-Seyl. Z.* **123**, 1.
—— & Kuhn (1923). *Hoppe-Seyl. Z.* **126**, 28.

92. LIVER ALDEHYDE OXIDASE

By A. H. GORDON, D. E. GREEN[1] and V. SUBRAHMANYAN

From the Biochemical Department, Cambridge

(*Received 27 March 1940*)

In the course of an attempt to isolate the xanthine oxidase of pig liver we observed that the ratio $\frac{\text{aldehyde activity}}{\text{xanthine activity}}$ dropped sharply after the first stages in the purification and then remained constant [cf. Corran *et al.* 1939, 1]. This initial fall in the ratio was interpreted at the time to mean that an enzyme inactive towards xanthine but active towards aldehydes was eliminated in the early purification of liver xanthine oxidase. Further investigation has now confirmed the presence in liver of an aldehyde enzyme other than the xanthine oxidase, and the present communication deals with its purification and properties. There have been hints in the literature of a specific aldehyde enzyme in liver [e.g. Lemberg *et al.* 1936] though no attempt was made to lend direct support to the claim by separating the aldehyde oxidase from the xanthine oxidase.

The aldehyde oxidase of liver is a flavoprotein whose prosthetic group is flavin adenine dinucleotide. The mechanism of the catalysis involves a cycle of reduction of the flavin group by aldehyde followed by oxidation of the leucoflavin by molecular oxygen. The enzyme contains another coloured group other than flavin though there is no information yet of its chemical nature.

(1) *Method of testing catalytic activity*

The aldehyde enzyme catalyses the oxidation of aldehydes by molecular oxygen and by other hydrogen acceptors such as methylene blue. Both the aerobic manometric method and the anaerobic Thunberg method are suitable for following enzyme activity but in practice we have relied on the anaerobic methylene blue technique.

We have arbitrarily defined as the unit of aldehyde activity an amount of enzyme that in presence of 0·1 ml. of *M* acetaldehyde will catalyse the reduction of 0·1 ml. of 0·0113 *M* methylene blue in 1 min. in *M*/25 phosphate buffer *p*H 7·2. The experiments were carried out at 38° in a total volume of 2·5 ml. The reduction time should not be greater than *ca.* 1 min. Beyond this limit the velocity is not strictly proportional to the activity of the enzyme. The mixture of enzyme and aldehyde was placed in the main tube and methylene blue in the side bulb. After 2 min. incubation at 38° the contents were rapidly mixed and the time required for complete decoloration of the methylene blue determined.

(2) *Method of preparation*

There are four main coloured impurities which must be eliminated before liver aldehyde oxidase can be obtained homogeneous from the point of view of coloured components: (1) haemoglobin, (2) xanthine oxidase flavoprotein, (3) a $Fe(OH)_3$-protein complex and (4) catalase. Haemoglobin offers little difficulty—being soluble in half-saturated $(NH_4)_2SO_4$ in contrast to the aldehyde enzyme which is completely precipitated at that saturation. Several $(NH_4)_2SO_4$

[1] Beit Memorial Research Fellow.

precipitations suffice to remove all traces of haemoglobin. The xanthine oxidase flavoprotein is largely destroyed by exposure in 25% alcohol to 48° for 5 min. whereas the aldehyde oxidase is stable under these conditions. The traces of xanthine oxidase which survive the heating procedure are removed in the course of further purification without any special efforts being necessary to attain this end. Pig liver contains a comparatively large amount of an orange red colloidal substance which behaves like a $Fe(OH)_3$-protein compound. This is probably identical with the so-called "ferratin". This $Fe(OH)_3$-protein is less soluble in $(NH_4)_2SO_4$ solutions than the aldehyde oxidase though the margin is slight. Some four or five fractionations in $(NH_4)_2SO_4$ solutions between the limits of 20 and 35% saturation are necessary before the enzyme can be completely separated from the $Fe(OH)_3$-protein. The fractionations are best carried out in ammoniacal $(NH_4)_2SO_4$ solutions for the following two reasons: (1) continued exposure of the $Fe(OH)_3$-protein to ammoniacal $(NH_4)_2SO_4$ leads to denaturation and the protein becomes insoluble even in absence of $(NH_4)_2SO_4$; (2) the aldehyde enzyme is unstable in slightly acid or neutral $(NH_4)_2SO_4$ but keeps indefinitely in ammoniacal solutions of $(NH_4)_2SO_4$. The elimination of catalase has proved to be the most difficult task. In fact we have not as yet been able to obtain a preparation of the aldehyde enzyme freed from all traces of catalase. Theoretically $(NH_4)_2SO_4$ fractionation should effectively separate aldehyde oxidase from catalase. The former is precipitated almost completely at one-third saturation of $(NH_4)_2SO_4$ whereas the latter is largely soluble at half saturation. One or two gross $(NH_4)_2SO_4$ fractionations do in fact remove more than 90% of the original catalase present. The residual catalase precipitates more or less within the same range of $(NH_4)_2SO_4$ concentrations as the aldehyde enzyme and as many as ten successive $(NH_4)_2SO_4$ fractionations have failed to accomplish any significant separation. The persistence with which catalase accompanies the aldehyde enzyme in the course of salt fractionations, adsorptions, solvent precipitations, cataphoresis etc. have led us to the view that catalase forms a type of compound with the aldehyde enzyme which cannot be resolved by the methods employed.

The following are the details of the method for preparing aldehyde enzyme in a highly purified state from fresh pig liver.

(1) 1·8 kg. of finely minced liver are mixed with 4 l. water and 1840 ml. 97% ethyl alcohol. The suspension is maintained at 48° for 5 min. and then rapidly cooled to 20° by addition of crushed ice. The heating must be so regulated that the thermometer reaches 48° within 4 min. of application of heat. The solution is heated in a round-bottom flask immersed in a boiling water bath. Stirring must be vigorous to prevent local overheating. The mixture is centrifuged for 10 min. and the precipitate of denatured protein is discarded.

(2) The clear, pale red supernatant fluid (5·6 l.) is treated with 40 ml. 25% basic lead acetate. The precipitate is centrifuged and decomposed by thorough shaking with 400 ml. of saturated Na_2HPO_4. The precipitate of lead phosphate is rejected. The supernatant fluid (570 ml.) is made 40% saturated with respect to $(NH_4)_2SO_4$. The precipitate is dissolved in 100 ml. water. The solution contains *ca.* 1100 units.

(3) Successive ammoniacal $(NH_4)_2SO_4$ fractionations are now employed to remove $Fe(OH)_3$-protein and catalase. The ammoniacal solution is prepared by adding 6 ml. of NH_4OH (sp. gr. 0·880) to 94 ml. of saturated $(NH_4)_2SO_4$. The fractionations are carried out between the limits of 27 and 40% saturation of $(NH_4)_2SO_4$. The flavoprotein which is associated with liver aldehyde oxidase activity is partially bleached by hydrosulphite whereas the colour both of

$Fe(OH)_3$-protein and catalase is not affected by hydrosulphite. The hydrosulphite test thus offers a quick and reliable test of which fraction contains least coloured impurity. If there is little change on addition of hydrosulphite the fraction is poor. If the bulk of the colour is bleached the fraction is good. Furthermore the residual colour after reduction gives a clue as to the coloured impurity present. A green residual colour confirms the presence of catalase; a reddish brown residual colour indicates $Fe(OH)_3$-protein.

The following are the details of a typical first fractionation:

Fraction	Units	
I	400	Reddish brown, largely $Fe(OH)_3$-protein
II	300	Yellow brown, some $Fe(OH)_3$-protein
III	300	Yellow brown, practically no $Fe(OH)_3$-protein
IV	75	Greenish yellow, some catalase
V	25	Green, catalase and colourless impurities

Fraction III contains least coloured impurity and is largely bleached by hydrosulphite. Fractions I, II and IV are now refractionated and the best fractions are amalgamated with fraction III. This procedure constitutes one complete $(NH_4)_2SO_4$ fractionation. At least three repetitions are necessary to effect complete separation of $Fe(OH)_3$-protein and maximum separation of catalase. The yield from 4 lb. of liver is *ca.* 250 units.

The method described above does not lead to the homogeneous enzyme. Our best preparations are probably of the order of 50 % pure.

(3) *Properties of purified enzyme*

Purified preparations of the enzyme are yellowish brown in colour. On reduction with hydrosulphite the colour is largely bleached and shaking with air restores the colour. In a later section evidence will be presented for the flavin nature of the enzyme.

The best preparations of the enzyme contain 0·17 % flavinphosphate. At this level of purity one enzyme unit is equivalent to 0·62 mg. dry weight. At 38° 1 mg. dry weight of the enzyme transfers 2700 μl. H_2 from acetaldehyde to methylene blue per hour ($Q_{M.B.}=2700$). Assuming that flavin represents the prosthetic group of the enzyme and there is one flavin group per molecule of enzyme, it follows that each molecule of the enzyme catalyses the oxidation of 520 molecules of acetaldehyde per min. at 38°. Table 1 compares the properties of the milk xanthine oxidase at the level of *ca.* 80 % homogeneity with those of the liver aldehyde enzyme prepared by the methods described.

Table 1. *Comparison of catalytic constants of liver aldehyde oxidase and milk aldehyde oxidase*

	Turnover no.	$Q_{M.B.}$	% flavin-phosphate	mg. per unit of enzyme activity
Liver*	520	2700	0·17	0·62
Milk†	570	4500	0·27	0·32

* At purity level represented by extinction ratio $\frac{E_{275\,m\mu}}{E_{450\,m\mu}}$ of 15·6.

† At purity level represented by extinction ratio of 6·2.

Purified preparations of the aldehyde enzyme show three absorption bands with maxima at 450, 380 and 280 mμ. The ratio of the extinctions at 280 and 450 mμ respectively is 15·5 in the best preparations. Fig. 1 shows the absorption spectrum of the enzyme at the extinction ratio 15·5 stage. The 450 mμ band

characteristic of flavin compounds is anomalous in that there is no trough on the violet end. There is clearly some interfering substance absorbing at *ca.* 390–440 mμ which accounts for the indefiniteness of the 450 and 360 mμ bands. Since catalase is known to be present in preparations of the enzyme at extinction ratio 15·5 stage and since catalase shows an intense absorption band with a maximum at 410 mμ we are inclined to the view that the deviation of the visible spectrum of the aldehyde enzyme from that of typical flavoproteins is referable to the catalase present in the preparation.

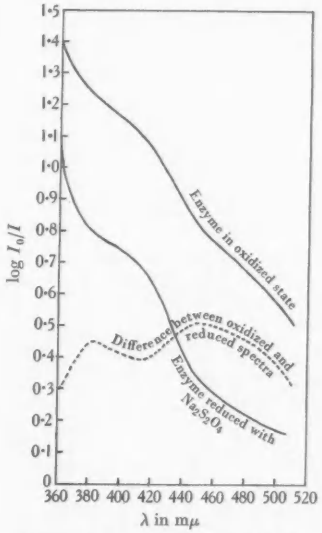


Fig. 1. Absorption spectrum of liver flavoprotein at ratio 15·5 stage. The enzyme solution contained 3·19 mg. per ml. and the length of the cell was 2 cm. For ease of presentation the ultraviolet end of the spectrum with the absorption band at 280 mμ is not shown in the figure.

A large variety of procedures has been explored with a view to advancing the purity of the enzyme beyond the ratio 15·5 stage. But this has so far not proved possible. The enzyme is remarkably stable in ammoniacal $(NH_4)_2SO_4$ but it cannot be further purified by constant repetition of the fractionation procedure. The enzyme is fragile in salt-free solution and in $(NH_4)_2SO_4$ solutions of neutral or acid reaction. Desiccation even at low temperatures completely inactivates the enzyme although no gross denaturation is detectable. Aqueous solutions of alcohol and acetone (> 40 %) rapidly denature the enzyme at room temperature though more slowly at 0°.

(4) *Evidence for flavoprotein nature of the enzyme*

The prosthetic group of liver aldehyde oxidase is flavin adenine dinucleotide. On boiling a solution of the enzyme the dinucleotide is liberated from the coagulated protein and can be estimated in the *d*-amino-acid oxidase test system

[cf. Straub 1938, and Corran *et al.* 1939, 2 for full details]. One unit of enzyme contains 1·1 μg. of flavin adenine dinucleotide. The ratio $\frac{\mu g.\ \text{flavin adenine dinucleotide}}{\text{enzyme unit}}$ remains fairly constant from the first clear extract to the highest purity level attained.

The flavin group can be split off from the enzyme by a variety of procedures, e.g. boiling, acidification to *p*H 4, or less, prolonged dialysis against water or dilute buffer, exposure to fat solvents etc. The splitting is irreversible in all these cases.

The following procedure was employed in the isolation of the dinucleotide [cf. Corran *et al.* 1939, 2]. A purified enzyme preparation was heated at 70° for 5 min. in presence of 15 % $(NH_4)_2SO_4$ and acetic acid (*p*H 3·8). After filtration the protein-free solution was saturated with $(NH_4)_2SO_4$ and extracted at *ca.* 70° with molten phenol. Water was added and the phenol was extracted with ether. The dinucleotide was then purified by precipitation as the Ag salt in dilute HNO_3 (*p*H 2). A solution of the decomposed Ag salt showed the typical absorption spectrum of flavin compounds with bands at 265, 370 and 450 mμ respectively. The ratio of the extinction of the three bands was 3·75 : 1·06 : 1 whereas the ratio for the pure dinucleotide is 3·2 : 0·8 : 1 [cf. Warburg & Christian, 1938]. Insufficient material set a limit to further purification of the flavin prosthetic group of the aldehyde enzyme but its identity with yeast flavin adenine dinucleotide could be established by another method. Comparison was made between the catalytic activities of the two dinucleotides in the *d*-amino-acid oxidase test:

	μl. O_2/10 min./μg. flavin phosphate
Yeast	132
Aldehyde oxidase (1)	135
Aldehyde oxidase (2)	126

Thus far no flavin compound other than flavin adenine dinucleotide has been found to be active in the *d*-amino-acid oxidase test.

On treatment of the enzyme with hydrosulphite the yellow-brown colour is only partially bleached. Flavin compounds whether free or combined with protein are completely bleached by hydrosulphite and indeed the prosthetic flavin group which has been separated from the protein part of the enzyme leaves no residue of colour after hydrosulphite treatment. It follows therefore that the flavoprotein must contain some coloured grouping other than flavin. From the extinction of the absorption band at 450 mμ and assuming a β value of $2{\cdot}4 \times 10^7$ as for other flavoprotein one can calculate how much flavin should be present in a given solution of the enzyme in order to account for the observed extinction. The flavin can then be split off from the enzyme and its concentration estimated spectrophotometrically. The results of many experiments agree that only 33 % of the total absorption at 450 mμ can be referred to flavin. The rest is due to some coloured group or groupings non-flavin in nature. It is interesting to note that Corran *et al.* [1939, 1] found that milk flavoprotein contained non-flavin coloured components accounting for the same proportion of the absorption at 450 mμ.

(5) *Mechanism of the reaction*

On addition of acetaldehyde to the enzyme under anaerobic conditions the enzyme is at once partially decolorized. On admission of air the colour is rapidly restored. The enzyme therefore undergoes a cycle of reduction by the substrate

and of oxidation by molecular oxygen. The question arises whether it is the flavin portion of the molecule which undergoes this cyclical process. Spectrophotometric analysis of the enzyme solutions reduced anaerobically by acetaldehyde shows that 33 % of the absorption at 450 mμ has disappeared. In other words the drop in light absorption corresponds precisely to the proportion of the total light absorption due to flavin. Furthermore subtraction of the absorption spectrum of the enzyme reduced with acetaldehyde from that of the oxidized enzyme yields an absorption spectrum of a flavin compound with bands at 450 and 380 mμ respectively. There can be no doubt therefore that it is the flavin group which is alternately reduced and oxidized and hence the justification for considering flavin adenine denucleotide as the prosthetic group of the enzyme.

The reduction of the enzyme by acetaldehyde under anaerobic conditions is for all practical purposes instantaneous. From the turnover number of 520 per min. it follows that the enzyme should be completely reduced in $\frac{1}{520}$ min. The great speed of the observed reduction offers in fact additional support of the cyclical mechanism of the catalysis.

Hydrosulphite bleaches the enzyme some 10 % more than acetaldehyde at 450 mμ. Part of the non-flavin groups must be therefore reducible by hydrosulphite but not by acetaldehyde.

(6) *Kinetics*

Fig. 2 shows how the rate of reduction of methylene blue by acetaldehyde varies with the concentration of enzyme. It is only over a restricted range of enzyme concentrations that a linear relation obtains. This fact must be taken into consideration in carrying out tests of enzyme activity for unless the reduction time of methylene is less than a minute a correction will have to be applied in calculating the number of enzyme units. This falling off in activity with increasing dilution of the enzyme is understandable in terms of destruction of the enzyme. The more dilute the enzyme the larger the time required to reduce a fixed amount of methylene blue. Assuming that the rate of destruction of the enzyme is a function of time only it follows that the ratio

$$\frac{\text{final active enzyme concentration}}{\text{initial active enzyme concentration}}$$

will steadily decrease with dilution of the enzyme. Consistent with this interpretation is the fact that the deviation from linearity progressively increases with dilution of the enzyme. The same phenomenon has been observed with the xanthine oxidase of milk [cf. Booth, 1935].

The relation between velocity and concentration of aldehyde is plotted in Fig. 3. The half-speed concentration of crotonaldehyde (K_m) is *ca.* 0·007 *M*. Crotonaldehyde was used in preference to acetaldehyde because of its higher boiling point which ensures that there will be no appreciable change in concentration during the evacuation of the Thunberg tubes.

The effect of temperature on the velocity of the reaction is shown in Fig. 4. Above 50° the rate of destruction begins to increase sharply and no simple temperature effect can be observed.

The enzyme is active over the *p*H range 5–11 with a maximum velocity at *ca.* *p*H 7 (cf. Fig. 5). The chemical composition of the buffer did not appear to affect the velocity for a given *p*H.

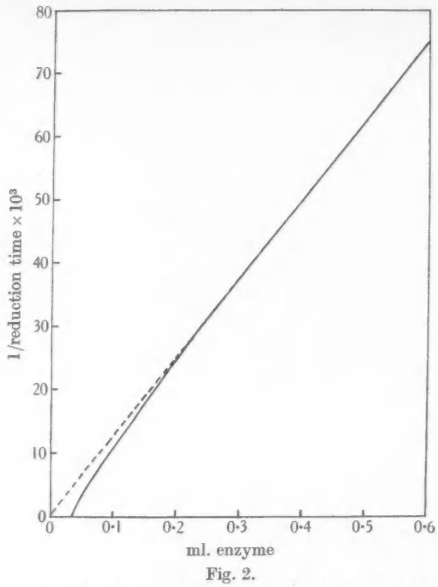


Fig. 2.

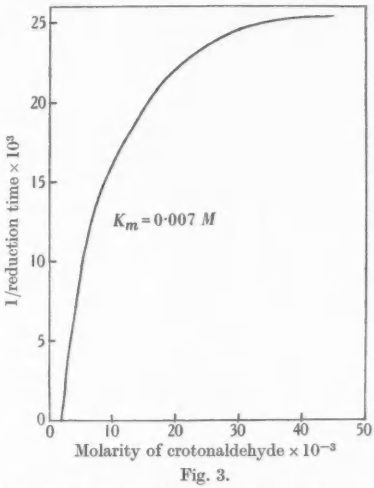


Fig. 3.

Fig. 2. Effect of enzyme concentration on velocity of reduction. The additions in each experiment were 1 ml. *M*/10 phosphate buffer, 0·1 ml. *M* acetaldehyde, 0·1 ml. 0·011 *M* methylene blue. Total volume, 2·5 ml. Reduction time in sec. The dotted line is the theoretical slope for a linear relation.

Fig. 3. Effect of aldehyde concentration. The additions in each experiment were 0·3 ml. enzyme, 1 ml. *M*/10 phosphate buffer and 0·1 ml. 0·011 *M* methylene blue.

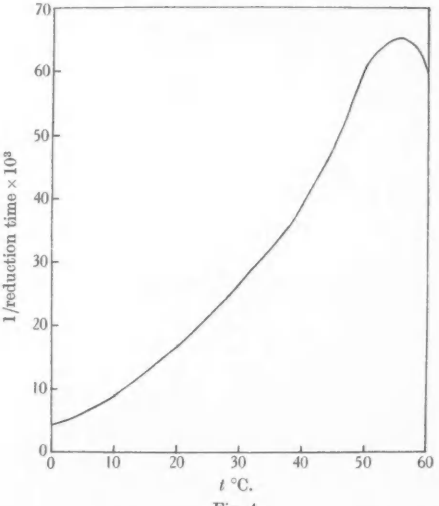


Fig. 4.

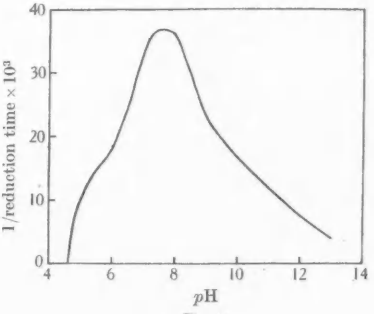


Fig. 5.

Fig. 4. Effect of temperature on velocity of reduction. The additions in each experiment were 0·2 ml. enzyme, 1 ml. *M*/10 phosphate buffer *p*H 7·2, 0·1 ml. 0·011 *M* methylene blue and 0·1 ml. *M* acetaldehyde.

Fig. 5. Effect of *p*H on velocity of reduction. The additions in each experiment were 0·2 ml. enzyme, 1 ml. buffer 0·1 ml. *M* acetaldehyde and 0·1 ml. 0·011 *M* methylene blue.

(7) *Specificity of substrate*

The liver aldehyde enzyme oxidizes a variety of aldehydes. Acetaldehyde, propaldehyde, butaldehyde, crotonaldehyde, benzaldehyde, salicylaldehyde and glycollic aldehyde have been tested and shown to be active as substrates (cf. Table 2). Among aliphatic aldehydes formaldehyde is an apparent exception, but in view of the fact that the enzyme is rapidly denatured in presence of formaldehyde the negative result does not necessarily exclude formaldehyde as substrate. Acetaldehyde is more active for a given concentration than propaldehyde. Increase in the length of the carbon chain decreases the velocity with which the aldehyde is attacked by the enzyme. The unsaturated aldehyde crotonaldehyde is oxidized as rapidly as acetaldehyde. Hydroxyaldehydes such as glycollic aldehyde and salicylaldehyde are less active than the corresponding unsubstituted aldehydes.

Ketones, ketonic acids, acids and alcohols cannot act as substrates. Hypoxanthine is active with crude preparations of the enzyme but this activity disappears when the higher purity levels are attained. The same applies to the oxidation of dihydrocoenzyme I.

Table 2. *Specificity of substrate*

Substrate	Final concentration	Relative activity (acetaldehyde as standard)
Acetaldehyde	*M*/10	100
Propaldehyde	*M*/10	17
Butaldehyde	*M*/10	13
Crotonaldehyde	*M*/10	66
Benzaldehyde	Saturated solution	25
Glycollic aldehyde	*M*/10	8
Salicylaldehyde	Saturated solution	2
Pyruvate	*M*/10	0
Ethyl alcohol	*M*/10	0

(8) *Specificity of hydrogen acceptor*

The enzyme catalyses the oxidation of aldehydes by molecular oxygen, oxidation-reduction indicators such as methylene blue, nitrate and cytochrome *c*. The catalytic reductions of nitrate and cytochrome *c* are comparatively slow reactions and are probably of little physiological interest. The reaction with oxygen is rapid and there can be little doubt that oxygen is the physiological hydrogen acceptor. Fig. 6 shows the rate of oxidation of crotonaldehyde by molecular oxygen as a function of time. Methylene blue in high concentration increases the velocity *ca.* 50%. The velocity with oxygen in the gas space is the same as with air.

(9) *Product of reaction*

When a limited quantity of aldehyde is allowed to react to completion with oxygen in presence of the enzyme the oxygen absorbed corresponds to 1 atom of oxygen per molecule of aldehyde (cf. Fig. 7). The theoretical oxygen uptakes are reached only with quantities of the order of 0·1 ml. *M*/10 aldehyde. With higher concentrations of aldehyde the reaction time is longer than 30 min. and the enzyme becomes inactivated before the reaction is complete. The stoichiometric proportions suggest that the aldehyde is oxidized to the corresponding acid:

(1) Crotonaldehyde $+ \frac{1}{2}O_2 \rightarrow$ crotonic acid.

The reaction between aldehyde and oxygen is not direct but proceeds through the intermediation of the enzyme in the following way:

(2) Crotonaldehyde + enzyme → crotonic acid + reduced enzyme.
(3) Reduced enzyme + O_2 → enzyme + H_2O_2.

We should expect therefore that each molecule of crotonaldehyde would react with two atoms of oxygen—the oxidation products being crotonic acid and

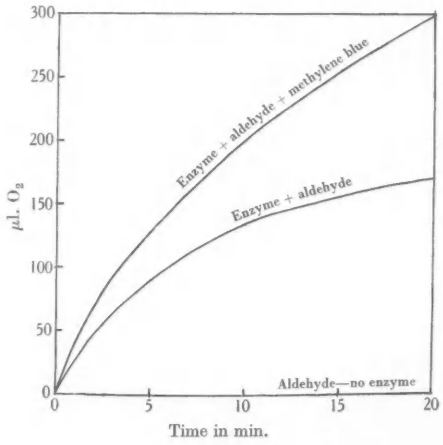


Fig. 6.

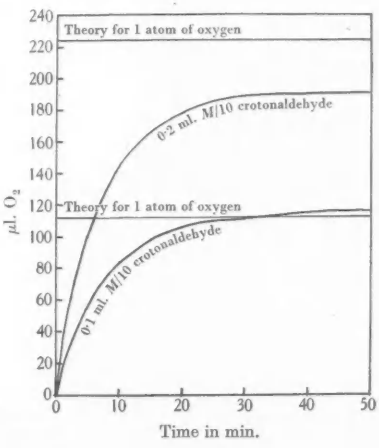


Fig. 7.

Fig. 6. The reaction of the enzyme system with oxygen in presence and absence of methylene blue. The additions were 1 ml. enzyme, 1 ml. *M*/10 phosphate buffer, 0·5 ml. *M*/10 crotonaldehyde and 0·2 ml. 0·5% methylene blue. Air in gas space, 38°.

Fig. 7. The oxygen equivalence of crotonaldehyde. The additions were 1·5 ml. enzyme, 0·5 ml. *M*/2 Na_2HPO_4 and 0·2 ml. 0·5% methylene blue.

H_2O_2. However the presence of catalase in the enzyme preparation ensures that all the H_2O_2 formed by autoxidation of the enzyme (or methylene blue) would be decomposed into O_2 and water:

(4) $2H_2O_2 \rightarrow 2H_2O + O_2$.

Reaction (1) therefore represents the balance of reactions (2), (3) and (4).

For the demonstration of the product of reaction we have carried out our experiments with acetaldehyde as substrate. A mixture containing 25 ml. enzyme, 27 ml. *M*/3 phosphate buffer *p*H 8, 3 ml. 0·5% methylene blue and 3 ml. *M* acetaldehyde was shaken aerobically at 38° for 60 min. From the oxygen uptake determined on an aliquot portion of the mixture 0·88 m*M* of acetic acid should have been formed. The distillate of the acidified mixture was found to contain 0·82 m*M* of acid and gave a positive reaction in the lanthanum nitrate test for acetic acid.

The method of Dyer [1916] was used for demonstrating that acetic acid was the only volatile acid in the distillate. The method involves steam distillation

at constant volume—the distillates being collected in 100 ml. lots and titrated against $N/10$ alkali. The following is an example of a typical experiment:

	Observed titre ml.	Theoretical titre ml.
1st 100 ml.	2·45	2·45
2nd 100 ml.	1·50	1·60
3rd 100 ml.	1·00	1·10

The values for the theoretical titres were determined in the apparatus with approximately the same quantity of pure acetic acid. The evidence is clear that acetic acid is the sole product of the oxidation.

(10) *Liver aldehyde oxidase and milk xanthine oxidase*

Xanthine oxidase of milk is a flavoprotein which catalyses the oxidation of purines, aldehydes and dihydrocoenzyme I [cf. Ball, 1939; Corran *et al.* 1939, 2]. The liver aldehyde oxidase is a flavoprotein with only one of the three functions of the milk compound. It is of considerable theoretical interest therefore to compare the properties of these two enzymes. We shall first list the similarities:

(*a*) Both contain in addition to flavin adenine dinucleotide some coloured substance or substances which account for some two-thirds of the light absorption at 450 mμ. In that respect they differ from all the other known flavoproteins.

(*b*) The catalytic efficiencies of the two enzymes with aldehydes as substrate are practically identical.

(*c*) Incubation with dilute cyanide and desiccation irreversibly inactivate the two enzymes as far as oxidation of aldehyde is concerned.

There are several differences other than that of substrate specificity:

(*a*) Whereas cyclical reduction and oxidation of the flavin group in the liver enzyme can be easily demonstrated there is no clear evidence of the operation of such a mechanism in the case of the milk enzyme.

(*b*) The liver enzyme is somewhat less soluble in $(NH_4)_2SO_4$ solutions than the milk enzyme—the precipitation range of the former being 25–35% saturation of $(NH_4)_2SO_4$ and that of the latter 33–43% saturation.

(*c*) The liver enzyme is rapidly and irreversibly inactivated by 24 hr. dialysis against distilled water at 0° whereas the milk enzyme is stable over a period of days.

The fact that milk flavoprotein apparently catalyses the oxidation of three entirely different substrates, viz. purines, aldehydes and dihydrocoenzyme I, has always been difficult to reconcile with the extreme specificity shown by enzymes in general. The isolation of a flavoprotein from liver which is specific for aldehyde oxidation and whose properties closely resemble its counterpart in milk gives new force to the suggestion that the milk enzyme may after all be a close association of three enzymes which cannot be resolved by the methods hitherto employed.

Liver contains at least three enzymes which catalyse the oxidation of aldehydes: (1) the aldehyde mutase of Dixon & Lutwak-Mann [1937]; (2) the xanthine-aldehyde-dihydrocoenzyme I oxidase of Corran *et al.* [1939, 2], and (3) the aldehyde oxidase described above. The first enzyme belongs to the class of the pyridinoproteins whereas the last two are flavoproteins.

REFERENCES

Ball (1939). *J. biol. Chem.* **128**, 51.
Booth (1935). *Biochem. J.* **29**, 1732.
Corran, Dewan, Gordon & Green (1939, 1). *Biochem. J.* **33**, 1694.
—— Green & Straub (1939, 2). *Biochem. J.* **33**, 793.
Dixon & Lutwak-Mann (1937). *Biochem. J.* **31**, 1347.
Dyer (1916). *J. biol. Chem.* **28**, 445.
Lemberg, Wyndham & Henry (1936). *Aust. J. exp. Biol. and med. Sci.* **14**, 259.
Straub (1938). *Nature, Lond.*, **141**, 603.
Warburg & Christian (1938). *Biochem. Z.* **298**, 368.

93. THE CITRIC ACID CYCLE AND THE SZENT-GYÖRGYI CYCLE IN PIGEON BREAST MUSCLE

By HANS ADOLF KREBS

From the Department of Biochemistry, University of Sheffield

(*Received 1 April 1940*)

It is known from Szent-Györgyi's work [1935; 1936] that oxaloacetate can be reduced by muscle tissue to form a mixture of fumarate and *l*(−)-malate. Szent-Györgyi formulates this reaction as follows:

(1) Oxaloacetate $\xrightarrow{+\text{"hydrogen of the substrates"}}$ {fumarate + *l*(−)-malate

So far two reactions which amplify the term "hydrogen of the substrates" have been described, viz.:

(2) "Triose" + oxaloacetate → pyruvate + {fumarate + *l*(−)-malate [Szent-Györgyi, 1936; Parnas & Szankowski, 1937]

and

(3) {Oxaloacetate + pyruvate} + oxaloacetate → citrate + CO_2 + {fumarate + *l*(−)-malate [Krebs & Johnson, 1937].

In this paper is described a third reaction in which oxaloacetate is reduced:

(4) Citrate + oxaloacetate → α-ketoglutarate + CO_2 + {fumarate + *l*(−)-malate

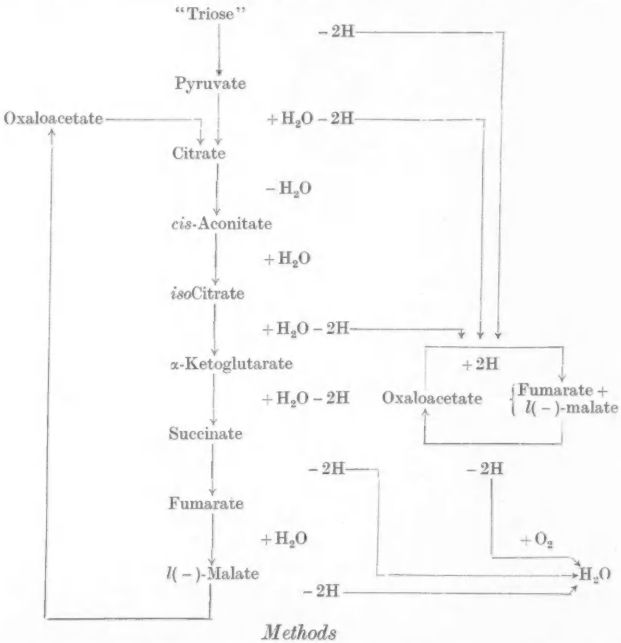
The reactions 2, 3 and 4 define the relations between the "citric acid cycle" and the "Szent-Györgyi cycle" in pigeon breast muscle. When one triose equivalent undergoes complete oxidation, 6 pairs of H atoms are released, in the course of the series of reactions shown in the right-hand section of the scheme on p. 776.

According to reactions 2, 3 and 4 the first 3 pairs of H atoms are transferred to oxaloacetate. They react later in the same way as the sixth pair (see below). The pathway of the fourth pair, arising from the dehydrogenation of α-ketoglutarate, is not yet clear. The fifth pair, arising from succinate, is known to react more or less directly with activated oxygen. The sixth and last pair, arising from *l*(−)-malate, reacts with activated oxygen through the intermediation of coenzymes. Thus the Szent-Györgyi cycle is concerned with the transport of (at least) 6 of the 12 H atoms released during the oxidation of one triose equivalent, as indicated by the scheme below. Since malate and oxaloacetate are not only catalysts, but also intermediates, the reaction malate $\xrightarrow{-2H}$ oxaloacetate occurs (at least) 4 times, and the reaction oxaloacetate $\xrightarrow{+2H}$ malate (at least) 3 times during the oxidation of a triose equivalent.

No attempt is made in this paper to describe the role of coenzymes in the aforementioned reactions, but it should be pointed out that reaction 4 presents a coenzyme problem of special interest. Adler *et al.* [1939] reported that *iso*citric dehydrogenase (which is responsible for the oxidation of citrate) requires the triphosphopyridine nucleotide (coenzyme II). Oxaloacetate on the other hand accepts H only from the diphosphopyridine nucleotide (coenzyme I). Neither of the two coenzymes therefore can act alone as a H carrier in reaction 4. One must assume that either both coenzymes together with an intermediate carrier

take part, or else that the transport of H by the coenzymes is coupled with their phosphorylation and dephosphorylation, resulting in an interconversion of the triphospho- into the diphospho-pyridine nucleotide. The possibility of such a reaction has been demonstrated by Euler & Bauer [1938] and by Adler *et al.* [1940].

Scheme of the oxidative breakdown of carbohydrate in pigeon breast muscle



Methods

The technique was in general the same as that used previously [Krebs & Eggleston, 1940]. Minced pigeon breast muscle was suspended in phosphate saline and shaken at 40° under varying conditions.

Citrate was determined according to Pucher *et al.* [1936]. The values obtained for citrate were multiplied with the factor 1·25 in order to include *iso*citrate and *cis*-aconitate with which citrate forms an equilibrium [Martius, 1938; Johnson, 1939]. The factor 1·25 follows from Johnson's data. Succinate was determined manometrically according to Szent-Györgyi & Göszy [1935] and Krebs [1937]. α-Ketoglutarate was treated at room temperature with an excess of permanganate, in the presence of $MnSO_4$ and H_2SO_4, and determined as succinate. Fumarate was determined by a new method worked out with the collaboration of Dr E. A. Evans and Dr D. H. Smyth. Fumarate is reduced to succinate in the presence of Zn and acid and the succinate is determined manometrically. The details of this method will be published later. The ratio fumarate/malate in the presence of fumarase was found to be 3·8 when equilibrium is established under the conditions of these experiments (40°, *p*H 7·4) [see Jacobsohn, 1934]. The concentration of malate was calculated on the basis of this figure.

Most experiments were carried out in triplicate or quadruplicate and the contents of one manometer cup (4 ml. muscle suspension) were used for determination of citrate, succinate, α-ketoglutarate and fumarate + malate respectively.

Oxidation of citrate in the presence of oxaloacetate

It will be seen from Table 1 that no appreciable quantities of citrate disappear when citrate alone is added anaerobically to muscle suspensions. When oxaloacetate and citrate are added together, however, citrate is removed and an

Table 1. *Oxidation of citrate in the presence of oxaloacetate*

Minced pigeon breast muscle; anaerobic conditions; 40°; muscle suspensions in 4 ml. phosphate-saline.

Exp.	Wet wt. muscle in 4 ml. mg.	Substrates added	Period of incubation min.	Changes in citrate μl.	α-Ketoglutarate formed μl.	Succinate formed μl.	Fumarate + malate formed μl.
1	266	1. 448 μl. citrate	50	+ 34	73	—	—
		2. 448 μl. citrate; 448 μl. oxaloacetate	50	−180	252	—	—
		3. 448 μl. citrate; 896 μl. oxaloacetate	50	−242	288	—	—
		4. 448 μl. oxaloacetate	50	—	52	—	—
		5. 896 μl. oxaloacetate	50	+ 25	100	—	—
		6. 448 μl. citrate; 448 μl. fumarate	50	+ 44	—	—	—
2	400	1. —	60	0	96	51	—
		2. 1344 μl. citrate	60	−274	195	25	—
		3. 1344 μl. oxaloacetate	60	—	128	114	—
		4. 1344 μl. citrate; 1344 μl. oxaloacetate	60	−780	697	89	—
3	400	1. 1344 μl. oxaloacetate; 1344 μl. citrate	10	−371	554	0	—
		2. 1344 μl. oxaloacetate; 1344 μl. citrate	20	−441	665	9	—
		3. 1344 μl. oxaloacetate; 1344 μl. citrate	40	−525	775	17	—
4	400	1. 1344 μl. oxaloacetate	15	+ 35	231	21	696
		2. 1344 μl. oxaloacetate; 1344 μl. citrate	15	−420	540	30	755
		3. —	15	+ 17	90	42	0
5	400	1. 1344 μl. oxaloacetate	40	+ 20	223	24	840
		2. 1344 μl. oxaloacetate; 1344 μl. citrate	40	−574	658	0	730

approximate equivalent of α-ketoglutarate is formed. The reaction occurs at relatively low concentrations of citrate and oxaloacetate (0·005 *M*). The rate of the reaction is very rapid: 400 mg. muscle (80 mg. dry wt.) remove 371 μl. citrate in 10 min. (exp. 3). If Q_{O_2} is 50 (the highest value observed in muscle) and if the total respiration passes through the citric acid cycle, the tissue should be capable of oxidizing at least 50/6 = 8·3 μl. citrate per mg. per hr. The observed figure (28 μl. per mg. dry wt. per hr.) is much higher than the postulated minimum.

The oxidation of citrate to α-ketoglutarate must be associated with an equivalent reduction. The only reductive process of sufficient magnitude accompanying the oxidation of citrate is the conversion of oxaloacetate into fumarate and malate. About 50–60 % of the added oxaloacetate is reduced to fumarate and malate. This figure is not essentially affected by the addition of citrate, especially if the period of incubation is comparatively long (40 min.). The formation of α-ketoglutarate, via reactions 3 and 4, is the oxidative equivalent of the reduction of oxaloacetate in the absence of added citrate.

Discussion of side issues

1. *Oxidation of α-ketoglutarate.* Attempts to demonstrate a dismutation between α-ketoglutarate and oxaloacetate

(5) α-Ketoglutarate + oxaloacetate + H_2O → succinate + CO_2 + $\left\{\begin{matrix}\text{fumarate} + \\ l(-)\text{-malate}\end{matrix}\right.$

have so far been unsuccessful, but the possibility that this reaction occurs in pigeon breast muscle cannot be excluded. If it occurs, two further H atoms of the triose equivalent pass through the Szent-Györgyi cycle.

In kidney cortex α-ketoglutarate is (chiefly) oxidized through the reaction [Krebs & Cohen, 1939]:

$$2\ \alpha\text{-Ketoglutarate} + NH_3 \rightarrow \text{glutamate} + \text{succinate} + CO_2.$$

2. *Reduction of fumarate to succinate.* The scheme of carbohydrate breakdown on p. 776 does not include the reaction fumarate → succinate. It is at present uncertain whether this reaction takes place and whether the system succinate⇌fumarate acts as a H-transporting system under physiological conditions. The reaction seems to occur when high concentrations of fumarate are present and when the conditions are anaerobic. Succinate is also found when oxaloacetate is added anaerobically to muscle tissue [see Krebs & Johnson, 1937; Krebs, 1940], but it is possible that it is not formed by reduction but oxidatively through the citric acid cycle, including reaction 5.

3. *α-Ketoglutarate formation from oxaloacetate.* The control experiments in Table 1 show that oxaloacetate yields anaerobically considerable quantities of α-ketoglutarate, a fact which is explained by the reactions 3 and 4. In previous papers [Krebs & Johnson, 1937; Krebs & Eggleston, 1940; Krebs, 1940], the formation of α-ketoglutarate was overlooked since the solution was treated with permanganate to remove malonate. The ketoglutarate therefore appeared as succinate in the results. As was previously pointed out the conclusions drawn from those experiments are not affected by the fact that part of the succinate found was derived from α-ketoglutarate.

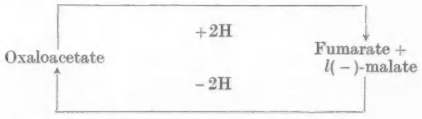
The oxidation of citrate by oxaloacetate accounts for the relatively low yields of citrate when tissues are incubated with oxaloacetate, i.e. when reaction 3 occurs [see Breusch, 1939; Thomas, 1939; Krebs, 1940].

Summary

It is shown that citrate and oxaloacetate react as follows in pigeon breast muscle:

$$\text{(4)}\quad \text{Citrate} + \text{oxaloacetate} = \alpha\text{-ketoglutarate} + CO_2 + \left\{\begin{matrix}\text{fumarate} + \\ l(-)\text{-malate}\end{matrix}\right.$$

Thus the Szent-Györgyi cycle



acts as a hydrogen carrier in the oxidation of citrate (reaction 4), in the formation of citrate [reaction 3; Krebs & Johnson, 1937] and in the oxidation of "triose" to pyruvate [reaction 2; Szent-Györgyi, 1936; Parnas & Szankowski, 1937]. The Szent-Györgyi cycle is therefore concerned with the transport of (at least) 6 of the 12 hydrogen atoms released during the oxidation of one triose equivalent, as shown in the scheme of carbohydrate oxidation on p. 776.

In other words, the oxidation of a triose equivalent involves one complete citric acid cycle and three repetitions of the Szent-Györgyi cycle.

This work was aided by grants from the Rockefeller Foundation and the Medical Research Council.

REFERENCES

Adler, Elliott & Elliot (1940). *Enzymologia*, **8**, 80.
—— von Euler, Günther & Plass (1939). *Biochem. J.* **33**, 1028.
Breusch (1939). *Biochem. J.* **33**, 1757.
von Euler & Bauer (1938). *Ber. dtsch. chem. Ges.* **71**, 411.
Jacobsohn (1934). *Biochem. Z.* **274**, 167.
Johnson (1939). *Biochem. J.* **33**, 1047.
Krebs (1937). *Biochem. J.* **31**, 2095.
—— (1940). *Biochem, J.* **34**, 460.
—— & Cohen (1939). *Biochem. J.* **33**, 1895.
—— & Eggleston (1940). *Biochem. J.* **34**, 442.
—— & Johnson (1937). *Enzymologia*, **4**, 148.
Martius (1938). *Hoppe-Seyl. Z.* **257**, 29.
Parnas & Szankowski (1937). *Enzymologia*, **3**, 220.
Pucher, Sherman & Vickery (1936). *J. biol. Chem.* **113**, 235.
Szent-Györgyi (1935). *Hoppe-Seyl. Z.* **235**, 1.
—— (1936). *Hoppe-Seyl. Z.* **244**, 105.
—— & Gözsy (1935). *Hoppe-Seyl. Z.* **236**, 54.
Thomas (1939). *Enzymologia*, **7**, 231.